REGULATORS OF TUMOR ANGIOGENESIS ARE MODULATED IN COLON CARCINOMA CELLS BY STRESS INFLICTED VIA NO AND PDTC

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1. Introduction

1.1 Cytokines and their Importance in Inflammatory Processes

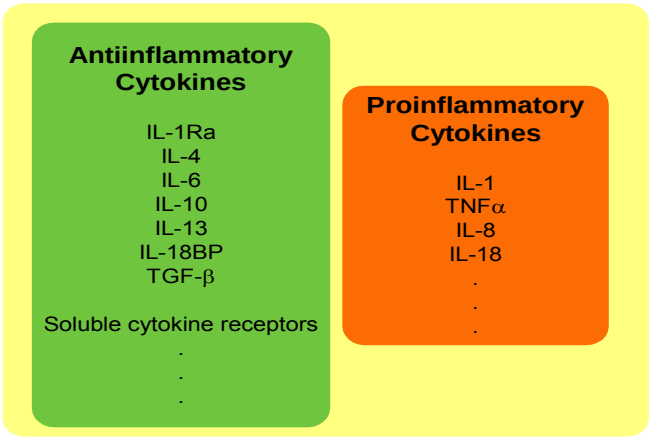
Cytokines are small proteins, often glycoproteins, with molecular weights ranging from 8 to 40 kD. They serve as biochemical messengers between cells (cytokine is derived from the Greek words for cell and kinesis) and are involved in processes such as regulation of the immune response (host response to disease or infection), stress responses, cell growth/differentiation, and tissue repair.
A single cytokine can exert numerous and various biological activities. Most of the cytokines are made by leukocytes and act on other leukocytes. That is the reason why they are also called interleukins (IL). But these mediators can also serve to communicate signals between immune and nonimmune cells so the name interleukin is not adjective.
Cytokines can be divided into four groups: α-helical cytokines (e.g. IFNγ), long-chain-β-sheet cytokines (e.g. IL-1 family and TNF family), α/β-cytokines (e.g. EGF family), and finally mosaic cytokines (e.g. IL-12). The maximal homology between selected cytokines in one group is not more than 20% to 30%. Several properties are shared by structurally diverse cytokines. Actions of cytokines can often be called pleiotropic and redundant. The ability of one cytokine to act on different cell types mediating diverse biologic effects is called pleiotropism. Synthesis and actions of cytokines are influenced by other cytokines. Several cytokines may interact to antagonize each other's action or to produce additive or synergistic effects. Additionally, the ability of one cytokine to induce the synthesis of others leads to the development of cytokine cascades. In these cascades the biologic effects may be mediated by a second or third cytokine of the upstream mediator. Cytokine actions can be locally or systemic. In most cases they act close to where they are produced in a autocrine or paracrine manner.
By binding to specific membrane receptors on target cells, cytokines initiate their function. To elicit biological effects only relative small quantities of a cytokine are sufficient. Different classes of cytokine receptors are known. Namely class I-IV cytokine receptors, the receptor kinase family, and the chemokine receptors. Receptor expression is an important regulatory parameter in cytokine biology. For example, increased expression of cytokine receptors is induced by stimulation of T or B lymphocytes with antigens. Receptor expression is also regulated by cytokines

themselves. This can be managed by other members of the cytokine family or even the same respective cytokine, permitting positive or negativ feedback. Cellular responses to most cytokines in general mediate changes in gene expression, resulting in new cellular functions which can be accompanied by proliferation or differentiation. Chemokines, which elicit rapid cell migration without new gene expression, and tumor necrosis factor (TNFα), which may induce cell death without requiring new protein synthesis are exceptions to this rule.

Secretion of cytokines is a short, self-limited event. A transient induction of *de novo* gene transcription is usually necessary to mediate their production. This process is a result of cellular activation by immune signals or stress. One reason for *de novo* gene transcription is the unstability of most messenger RNAs encoding cytokines. The production of some cytokines may be controlled by RNA processing and additionally by posttranscriptional mechanisms, such as proteolytic release of an active product from an inactive precursor (e.g. pro-IL-1β). Due to their influence on inflammatory processes cytokines can be divided into two groups.

The most important immunoregulatory cytokines are listed in the following table:

Figure 1



First the proinflammatory cytokines which have the potential to make inflammatory disease worse. Second the antiinflammatory cytokines which serve to reduce inflammation and promote healing. The balance of pro- and antiinflammatory cytokines is dynamic. It is an ever-shifting process in which the amount of one cytokine-type can determine the kinetic and outcome of an inflammatory response. The human immune response is also regulated by specific cytokine inhibitors and soluble cytokine receptors. Furthermore, the net effect of any cytokine is dependent on several factors: the timepoint of cytokine release or the local milieu in which it

acts, the presence of competing or synergistic elements, cytokine receptor density, and tissue responsiveness to each cytokine. (Dinarello C.A. and Moldawer L.L., 2000; Janeway C.A. and Travers P., 1997; Abbas A., 2000)

1.1.2 Proinflammatory and Antiinflammatory Cytokines

As explained above, cytokines can be divided into proinflammatory and antiinflammatory cytokines. In healthy persons proinflammatory cytokines are produced at low levels. When they are secreted at higher levels they induce a cascade of gene products. Such genes can be for example the type II A phospolipase (PL) A_2, inducible nitric oxide (NO) synthase (iNOS), adhesion molecules, and IL-8. Enzymes for which the genes are coding are responsible for an increased synthesis of prostaglandins, leukotrienes, NO, O_2^-, matrix metallo-proteinases (MMPs), and other mediators of inflammation. Consequences of an increased level of these mediators of inflammation are the development of the cardinal symptoms of inflammation: redness, heat, swelling, pain, and loss of function. IL-1, TNFα and IL-18 are initiators of this cascade of inflammatory mediators. These initiators are induced by infection, tissue damage, ischemia, immune-activated T cells, or toxins. For example the cytokines IL-12 and IL-18 are primarily secreted after microbial infection. This process leads to an induction of proinflamatory cytokines and IFNγ, which is known to direct T cell development along a T_H1 pathway. Proinflammatory cytokines activate important regulators of gene transcription (e.g.: NF-κB, AP-1, NF-IL-6). Their overproduction is a frequent characteristic seen in human autoimmune diseases such as Crohn's disease, lupus erythematosus, and rheumatoid arthritis.

As mentioned above, there are antiinflammatory cytokines which are involved in controlling the proinflammatory cytokine response. The antiinflammatory cytokines are able to suppress genes coding for proinflammatory cytokines such as IL-1, TNF, and chemokines. In the presence of IL4 and IL-10 activated naive T cells develop along the T_H2 pathway. Secreted antiinflammatory binding proteins interfere with the action of proinflammatory cytokines on the level of bioactivity (Dinarello C.A., Moldawer L.L:, 2000).

1.1.3 Adaptive Immunity and the Role of T cell cytokines

Infectious agents induce the very complex defense mechanism of adaptive immunity. The type of immunity is developed out of a response to an infection and is individually adapted to a given specific kind of infection. Magnitude and defensive capabilities are increased with each successive exposure to a particular antigen. $CD4^+$ helper T cells respond to the protein antigens of microbes and may differentiate into special subsets of helper T cells called T_H1 and T_H2 cells. The differentiation into the various T helper cells is influenced primarily by the cytokines present during the initial proliferative phase of T-cell activation. This process not only determines the T cell effector function, but also the participation in the development and expansion of the respective subsets. For example, IFN-γ secreted by T_H1 cells promotes further T_H1 differentiation and inhibits the proliferation of T_H2 cells in parallel. For cell-mediated immune responses the most important differentiation-inducing stimuli are IFN-γ, IL-2, IL-12, and IL-18 being the major inducers of T_H1 cells. T_H1 differentiation is stimulated by intracellular bacteria, such as *Listeria* and mycobacteria, and by some parasites, such as *Leishmania*. These are all microorganisms which can infect macrophages. Many organ-specific autoimmune diseases and inflammatory reactions such as granulomas are due to excessive activation of T_H1 responses. In contrast, T_H2 development is promoted by stimulation of activated naive cells in the presence of IL-4 and IL-10. Both interleukins produced by T_H2 cells inhibit activation of T_H1 cells. So the most important differentiation-inducing stimuli for humoral responses are the cytokines IL-4 and IL-10 produced by T_H2 cells. T_H2 differentiation occurs in response to helminth and allergens, which cause chronic T cell stimulation, often with little macrophage activation. The principal effector function of T_H2 cells is in IgE and eosinophil/mast cell-mediated immune reactions. The balance between cytokine signals regulates the universal pattern of the aquired immune response. Once determined, the development into a T_H1 or T_H2 cell population amplifies itself and cross inhibits the other way. Chronic immune reactions are often dominated by either T_H1 or T_H2 populations. T cell differentiation is determined by stimuli present early during immune responses. The induction of IFN-γ is dominated by IL-12 and IL-18, produced by accessory or antigen presenting cells such as macrophages or

dendritic cells, thereby linking acquired and innate immune responses. (Janeway C.A. and Travers P., 1997; Dinarello C.A. and Moldawer L.L:, 2000).

1.1.4 Interferon-γ

Activated T cells and natural killer (NK) cells produce interferon-γ (IFN-γ), a pleiotropic cytokine involved in antiproliferative and antiviral responses, immune surveillance, and tumor suppression (Stark, G.R. et al, 1998; Bach E. et al, 1997). lFN-γ is the major means by which T cells activate macrophages. The complex genetic programs elicited by lFN-γ in the immune system, account for the diverse activities of IFN-γ in mediating host defense and immunopathology. Biological responses to IFN-γ are mediated mainly by regulation of gene expression. It has been established that the majority of the pleiotropic effects of IFN-γ is owing to several gene products that are regulated by the Jak-Stat 1 (signal transducer and activator of transcription 1) pathway (Darnell J.E., Jr et al, 1994)

1.1.4.1 IFN-γ and immunity

IFN-γ is a homodimeric glycoprotein with pleiotropic immunologic functions in cell-mediated immunity against intracellular microbes. IFN-γ can promote the microbicidal function of macrophages by stimulating the synthesis of reactive oxygen intermediates (ROI) and NO. These effects are mediated by activating transcription and/or assembly of the enzymes phagocyte oxidase and iNOS. These radicals serve to destroy microbes contained within phagolysosomes. IFN-γ enhances major histocompatibility complex class (MHC)-associated antigen presentation and amplifies the recognition phase of immune responses by increasing expression of ligands that are recognized by T cells. This cytokine also activates vascular endothelial cells and potentiates many of the actions of TNFα on endothelial cells, thereby promoting T lymphocyte adhesion and extravasation to sites of infection. The T_H1 inducing effect of IFNγ is mediated indirectly by activating mononuclear phagocytes to produce IL-12, the prototype of T_H1 cytokines. In mice IFN-γ also enhances expression of the signaling chain of the IL-12 receptor. IFN-γ induces

antibody responses that also participate in phagocyte-mediated elimination of microbes. The B-cell IgG subclasses (IgG2a and IgG3) induced by IFN-γ bind to Fcγ receptors on phagocytes and activate complement. Both of these mechanisms promote the phagocytosis of opsonized microbes. Furthermore, IFN-γ activates neutrophils and enhances the cytotoxicity of NK cells (Janeway c.a. and Travers P., 1997). The absence of IFN-γ production or cellular responsiveness in humans and experimental animals significantly predisposes the host to microbial infections. Mice with a targeted disruption of the IFN-γ gene have been developed by homologous recombination in ES cells. These animals develop normally and are healthy in the absence of pathogens. They are characterized, however, by an impaired production of macrophage antimicrobial products and reduced expression of macrophage MHC class II antigens. After infection, IFN-γ-deficient mice die by normally sublethal doses of the intracellular pathogen Mycobacterium bovis. Resting splenic natural killer cell activity is reduced in these mice (Dalton D.K., 1993). These observations validate the pathophysiological importance of this cytokine in preventing infectious diseases.
In addition to the aforementioned role of IFN-γ in immune defence against infection, IFN-γ has been characterized as a potent tumorsuppressive cytokine. The IFN-γ signaling pathway leads to apoptosis and to the expression of immune modulatory proteins (e.g. IP-10, MIG and iNOS) that can cooperate with T cells in the destruction of tumors. Various lines of experimental approach have, in general, supported the hypothesis that the IFN-γ signaling pathway is anti-tumorigenic (Ikeda H., et al, 2002). In spite of the antiproliferative and tumorsuppressive activities of IFN-γ its use in the treatment of various malignancies has so far been disappointing. In many instances a combination treatment with other interferons, and also with chemotherapy was moderately effective only (Digel W et al 1991).

1.2 Chemokines

Chemokines are cytokines which can induce chemotactic reactions of cells. They are small proteins that are potent activators and chemoattractants for leukocyte subpopulations and some nonhematopoietic cells. Their actions are mediated by a family of seven transmembrane G protein-coupled receptors, the number of which has grown considerably in recent years. Significant advances have been made in

understanding the regulation of chemokine receptor expression and the intracellular signaling mechanisms used in bringing about cell activation. Chemokine receptors have also recently been implicated in several disease states including allergy, psoriasis, rheumatoid arthritis, atherosclerosis, and malaria. However, most fascinating has been the observation that some of these receptors are used by human immunodeficiency virus type 1 in gaining entry into permissive cells (Murdoch C. and Finn A., 2000).

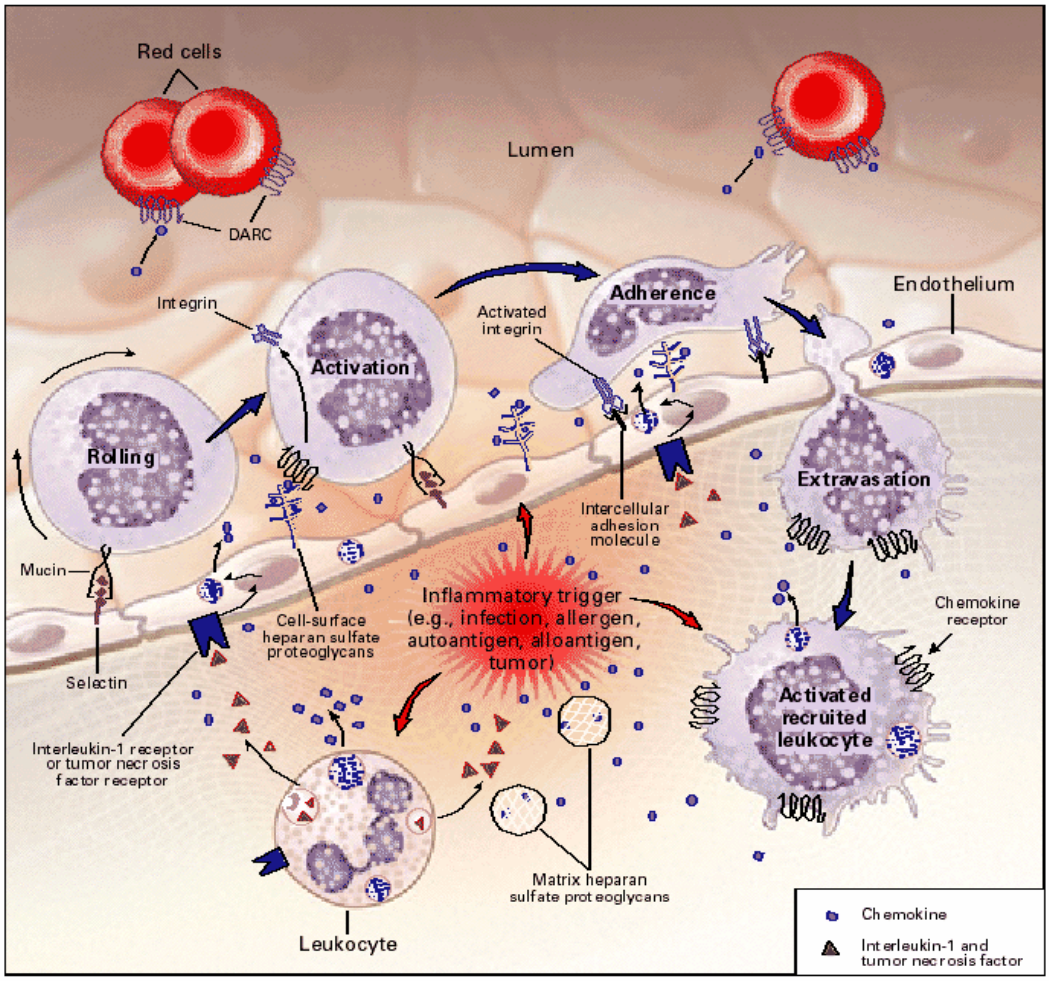


Fig. 2: Chemokines and Leukocyte Movement.

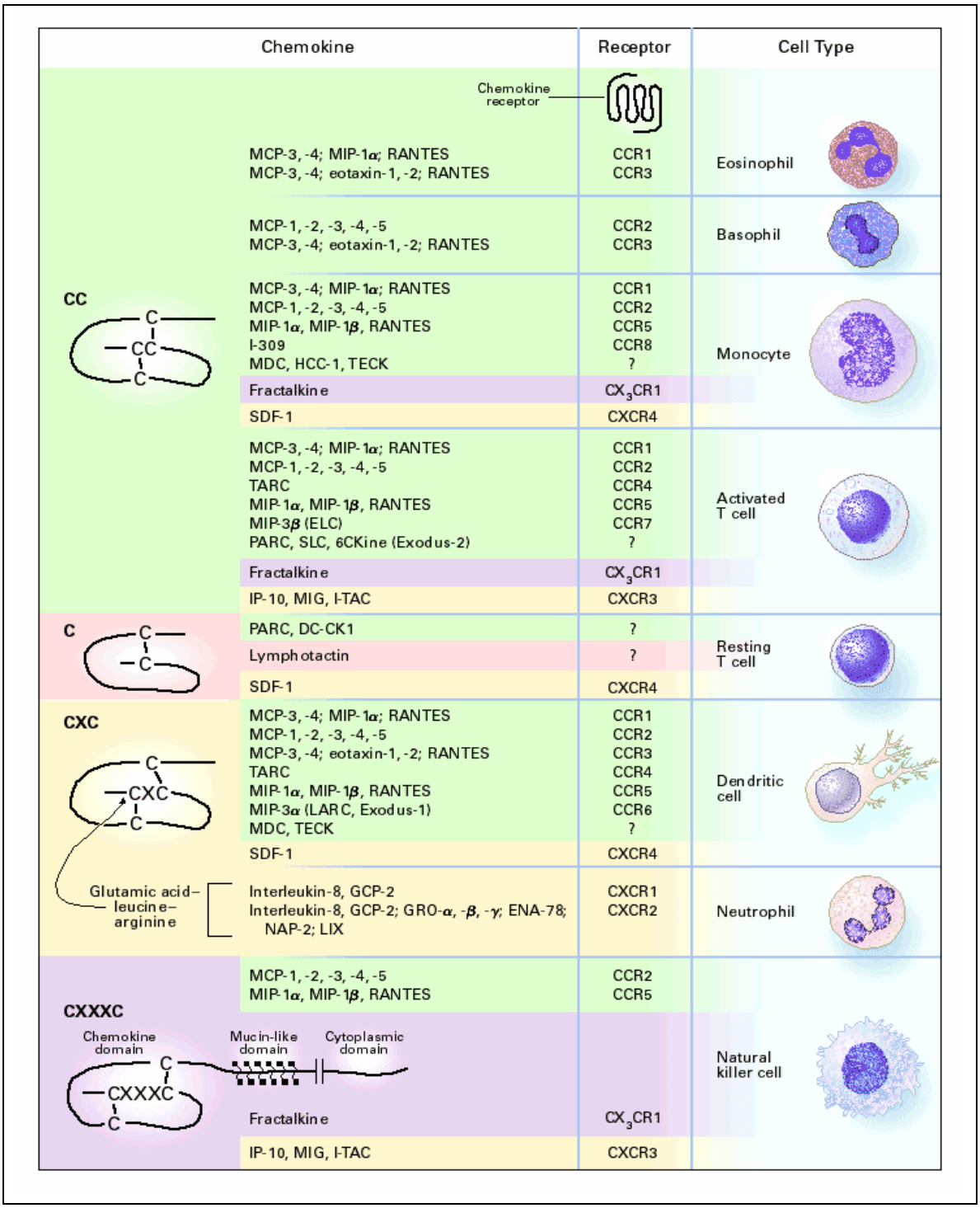
Chemokines are secreted at sites of inflammation and infection by resident tissue cells as well as, resident and recruited leukocytes. Chemokines are locally retained on matrix and cell-surface heparan sulfate proteoglycans. An established chemokine concentration gradient is surrounding the inflammatory stimulus. Leukocytes rolling on the endothelium in a selectin-mediated process are brought into contact with chemokines recruiting cell-surface heparan sulfate proteoglycans. Chemokine signaling activates leukocyte integrins, leading to firm adherence and extravasation. The Duffy antigen receptor for chemokines (DARC), a promiscous erythrocyte chemokine receptor, functions as a sink, removing chemokines from the circulation and thus helping establishing a tissue-bloodstrearn chemokine gradient (adapted from Luster A.D., 1998).

Phagocytic leukocytes of the immune system undergo rapid and directed movements in chemoattractant gradients, a property that enables them to serve as the first line of cell-mediated host defense against infection. The interaction of chemoattractants with leukocytes initiates a series of coordinated biochemical and cellular events that include alterations in ion fluxes, integrin avidity as well as transmembrane potential, changes in cell shape, secretion of lysosomal enzymes, production of superoxide anions, and enhanced locomotion.

Two groups of chemoattractants have been identified and extensively studied. The first group consists of "classical" chemoattractants, such as bacterial-derived N-formyl peptides, the complement fragments C5a and C3a, and lipid molecules such as leukotriene B_4 and platelet activating factor. These are all chemoattractants and activators of leukocytes (Schiffmann E. and Corcoran B.A., 1975; Gerard C. and Gerard N.P.,1994; Goldman D.W. and Goetzl E.J., 1982; Hanahan D.J., 1986). The second group consists of molecules which have been detected during the last 10 years. A number of chemotactic cytokines in the 8- to 17-kD molecular mass range have been shown to be selective chemoattractants for leukocyte subpopulations *in vitro* and to elicit the accumulation of inflammatory cells *in vivo* (Baggiolini M. et al,1994 and 1997). These chemotactic cytokines belong to the chemokine superfamily, which can be divided into 4 groups (CXC, CX_3C, CC, and C) according to the positioning of the first 2 closely paired and highly conserved cysteines in the amino acid sequence. The specific effects of chemokines on their target cells are mediated by members of a family of seven transmembrane spanning G protein-coupled receptors (Murphy PM.,1994). These chemokine receptors are part of a much larger superfamily of G-protein-coupled receptors that include receptors for hormones, neurotransmitters, paracrine substances, inflammatory mediators, proteinases, taste and odorant molecules, and even photons and calcium ions (Watson B., 1994).

Fig. 3: Chemokines and their Detectors
The chemokines are 8-to-10-kD proteins that are subdivided into families on the basis of the relative position of conserved cysteine residues in the mature protein. In the α-chemokines, the first two cysteine residues are separated by a single amino acid (CXC), whereas in the β-chemokines, these two cysteine residues are adjacent to each other (CC). The α-chemokines that contain the sequence glutamic acid-leucine-arginine preceding the CXC sequence are chemotactic for neutrophils, and those that do not contain this sequence act on lymphocytes. The C chemokine lymphotactin has only two cysteines in the mature protein, and the CXXXC chemokine fractalkine has three amino acids separating the first two cysteines. Chemokine receptors are G protein-coupled proteins that are expressed on subgroups of leukocytes. Four human CXC chemokine receptors (CXCR1 through CXCR4), eight human CC chemokine receptors (CCR1 through CCR8), and one human CXXXC chemokine receptor (CX3CR1) have been identified so far. MCP denotes monocyte chemoattractant

protein, MIP macrophage inflammatory protein, RANTES regulated upon activation normal T-cell expressed and secreted, MDC macrophage-derived chemokine, HCC-1 hemofiltrate CC chemokine, TECK thymus-expressed chemokine, SDF-1 stromal-cell-derived factor 1, TARC thymus and activation-regulated chemokine, ELC EBI1 (Epstein-Barr virus-induced gene 1) ligand chemokine, PARC pulmonary and activation-regulated chemokine, SLC secondary lymphoid tissue chemokine, 6Ckine 6-cysteine chemokine, IP-10 interferon-inducible protein l0, MIG monokine induced by interferon-γ, I-TAC interferon-inducible T-cell alpha chemoattractant, DC-CK1 dendritic-cell chemokine 1, LARC liver and activation-regulated chemokine, GCP granulocyte chemotactic protein, GRO growth-regulated oncogene, ENA-78 epithelial-cell-derived neutrophil-activating peptide 78, NAP-2 neutrophil-activating peptide 2, and LIX lipopolysaccharide-induced CXC chemokine (adapted from Luster, A.D., (1998)).



1.2.1 Chemokines in Cancer

Chemokines modulate tumor behaviour by three main mechanisms: regulation of tumor-associated angiogenesis, activation of the host's tumor-specific immunological response, and direct stimulation of tumor cell proliferation in an autocrine fashion. All of these mechanisms are promising targets of cancer intervention, and preclinical mouse models suggest that certain chemokine antagonists and agonists could become important in the development of new anticancer therapies (Belperio J.A., et al. 2000).

1.2.1.1 Chemokines modulate angiogenesis

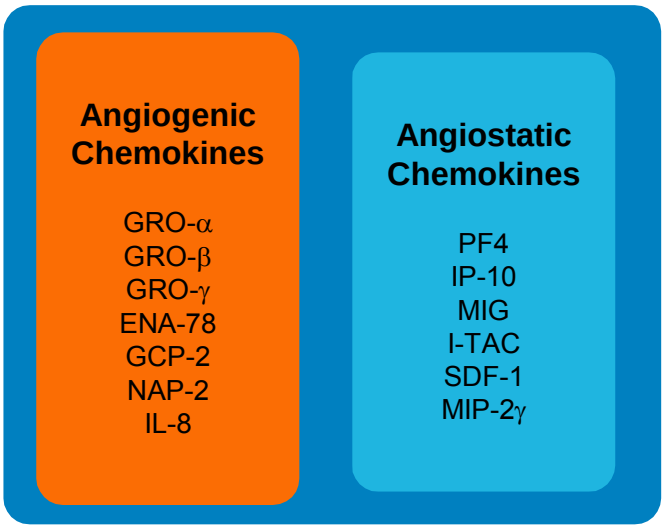
Angiogenesis is the growth of new blood vessels from already existing vessels and microcapillaries (Norrby K., 1997). It is a process that normally takes place during embryonic development and wound healing, but is also required for solid tumors to grow beyond 2 mm in diameter (Norrby K., 1997; Folkman J., 1995). Vascularisation of a tumor is required for rapid growth (Folkman J., 1995).

1.2.1.1.1 Imbalance of chemokines modulating angiogenesis regulates tumor growth

The Far Eastern concept of 'Yin and Yang' (i.e. that everything is controlled by two equal but opposing forces) is a recurring motif throughout biology, as processes such as angiogenesis must be temporally turned on and off during the life of an organism. It has been proposed that angiogenesis is regulated by a complex balancing act between opposing angiogenic and angiostatic factors (Moore B.B., et al, 1998). Considerable experimental evidence suggests that this hypothesis is valid. Much of the data are supporting a role for CXC chemokines in modulating the angiogenesis of tumors in vivo (Belperio J.A. et al, 2000). For example the participation of IL-8 in macrophage-mediated angiogenesis is suggested by the observation that IL-8 antisense RNA blocks monocyte-induced angiogenic activity (Koch A.E., et al, 1992). In contrast to the proangiogenic IL-8, the chemokines IP-10 and MIG are potent antiangiogenic mediators. Tumor cells genetically engineered to express high levels

of murine IP-10 have been shown to elicit a powerful host-mediated antitumor effect in vivo which appears to be mediated by the recruitment of inflammatory infiltrates composed of lymphocytes, neutrophils, and monocytes. Notably, IP-10 has been shown to be a potent endogenous inhibitor of angiogenesis (Angiolillo A.L., et al, 1995). Moreover, injection of recombinant human MIG into Burkitt tumors growing subcutaneously in nude mice causes tumor necrosis associated with extensive vascular damage (Sgadari et al, 1997). Finally, high levels of MIG and IP-10 gene expression were reported in tissues with necrosis and vascular damage in EBV positive lymphomatoid granulomatosis and nasal-type T/natural killer cell lymphomas (Teruya-Feldstein et al, 1997). The following table shows chemokines that modulate angiogenesis:

Figure 4



1.2.1.2 Chemokines can activate tumor-specific immunity

One of the greatest challenges in developing tumor vaccines is getting the host to recognize tumors that might be poorly immunogenic for a number of reasons. There is considerable experimental evidence that certain chemokines, particularly those from the CC family, are able to activate a tumor-specific immune response capable of mediating rejection of ordinarily 'non-immunogenic' cancers. These are for example MIP-1α, ELC, LARC and SLC (Mitchell J., et al, 2001). This is particularly significant, as one of the major obstacles in using immunotherapy to treat cancer has been the low immunogenicity of spontaneously arising human tumors (Bodey B., et al, 2000). Certain members of the CC chemokine family are chemotactic for either monocytes

and dendritic cells (antigen-presenting cells) or T cells (including cytotoxic T cells, and T helper 1 cells). These cell types are of major importance in generating specific immunity. An example demonstrating that chemokines can activate tumor-specific immunity are experiments with mice that are able to reject a colon adenocarcinoma cell line only if it has been engineered to express macrophage inflammatory protein 1α (MIP-1α/CCL3). This chemokine is chemotactic for monocytes, T cells and dendritic cells (Nakashima E., et al, 1996). Mice that had successfully rejected the MIP-1α transfected tumor cells are protected from subsequent inoculation with the parental unmodified adenocarcinoma cells. The rejected tumors are highly infiltrated by macrophages and neutrophils. Immune modulatory factors produced in the microenvironment within a tumor can determine whether or not an immune response will take place. Chemokines can influence production of these factors by recruiting and activating cytotoxic T cells and NK cells.

1.2.2 Chemokines as autocrine growth factors

Autocrine growth factors are proteins that can bind to receptors on the same cell that also produce them and that can stimulate cell proliferation. The GRO CXC chemokines were originally called 'melanoma growth stimulatory activity' polypeptides because these proteins are mitogenic for malignant melanoma cell lines *in vitro*. GRO-α was initially isolated from conditioned supernatant of the melanoma cell line Hs294T (Richmond A., et al, 1988), and is now known to be produced by many melanoma tumors. The significance of GRO chemokine expression in melanoma has been examined (Luan J., et al, 1997). Both GRO-α and its receptor CXCR2 are commonly expressed in malignant melanoma biopsies as assessed by immunohistochemistry. GRO-α and GRO-γ are expressed by melanoma tumors *in vivo*, but expression of the alpha form tends to be higher. As antibodies to GRO-α were found to inhibit *in vitro* growth of melanoma cell lines, GRO-α fits all the criteria for an autocrine growth factor. GRO-α has also been shown to function as an autocrine growth factor for certain adenocarcinoma cell lines derived from lung and stomach (Fujisawa N., et al, 2000).
Many tumors have been shown to overexpress IL-8, compared with their normal, non-malignant tissues. IL-8 is an autocrine growth factor for melanomas

(Schadendorf D., et al, 1993) as well as for certain tumor cells derived from cancers of the colon, stomach, liver, pancreas and skin (Fujisawa N., et al, 2000; Brew R., et al. 2000; Miyamoto M., et al, 1998; Metzner B., et al, 1999). In human biopsy tissues from ovarian carcinomas, neuroblastomas, and squamous cell carcinomas of the head and neck, both IL-8 and its receptor are expressed by cancerous cells, suggesting that IL-8 may also function in an autocrine loop in these tumors (Ivarsson K., et al, 2000; Ferrer F.A., et al, 2000; Richards B.L., et al, 1997). The autocrine functions of GRO-α and IL-8 illustrate the pleiotropism of chemokines, as these particular molecules not only stimulate tumors to multiply, but also recruit and expand endothelial cells *in vivo* to ensure that tumors develop an adequate vasculature as they grow. Thus, GRO-α and IL-8 are the most ideal tumor growth factors.

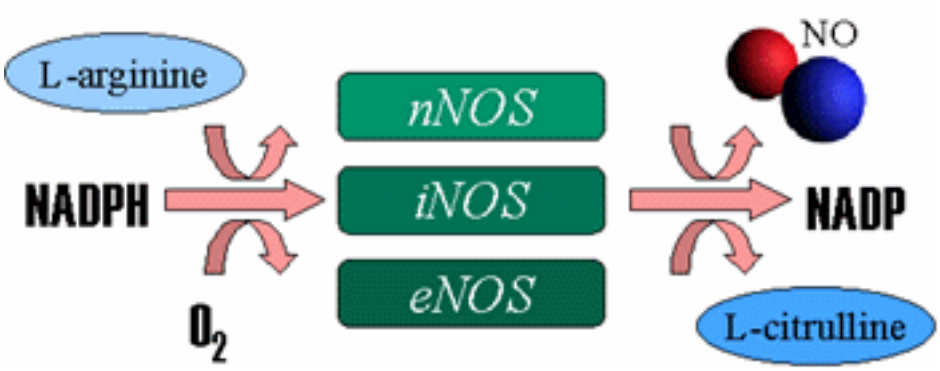
1.3 Nitric Oxide (NO)

NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes including vasodilation , neuronal function, inflammation and immune function. Its role was first discovered by several groups who were attempting to identify the agent responsible for promoting blood vessel relaxation and regulating vascular tone. This activity was termed endothelium-derived relaxing factor (EDRF). The discovery that EDRF was in fact nitric oxide - a small gaseous molecule - has led to an explosion of interest in this field. Nitric oxide has now been demonstrated to play a role in a variety of biological processes including neurotransmission, immune defence, and regulation of apoptotic cell death. Nitric oxide is a fairly short-lived molecule (with a half-life of a few seconds) produced by enzymes known as nitric oxide synthases (NOS). NO diffuses rapidly across cell membranes and, depending on the conditions, is able to cover a distance of several hundred microns. The biological effects of NO are mediated through the reaction of NO with a number of targets such as haem groups, sulfhydryl groups and iron and zinc clusters. Such a diverse range of potential targets for NO explains the large number of systems that utilize it as a regulatory molecule. Accordingly, abnormal regulation of NO synthesis is capable of affecting a number of important biological processes and has been implicated in a variety of diseases (Kolb J.P., 2000).

1.3.1 Nitric Oxide Synthases (NOS)

NO is produced by a group of enzymes called nitric oxide synthases. These enzymes convert arginine and O_2 into citrulline and NO. NADPH is a necessary co-factor. There are three isoforms of nitric oxide synthase (NOS) named according to the tissue type in which they were first described. The isoforms of NOS are neuronal NOS (or nNOS), endothelial NOS (or eNOS) and inducible NOS (or iNOS). All three isoforms can be found in a variety of tissues and cell types. The general path of NO production by NOS is illustrated in Figure 5:

Figure 5:



Two of the enzymes (nNOS and eNOS) are constitutively expressed in mammalian cells and synthesize NO in response to increases in intracellular calcium levels. Activation of iNOS activity is not directly mediated by Ca^{2+}, but is rather regulated on the level of gene transcription. Like all of the NOS isoforms – iNOS activation is dependent on the binding of calmodulin (CaM). In case of iNOS, CaM is irreversibly bound to the enzyme and the activity of the enzyme is regulated by its rate of expression rather than by the Ca 2+ concentration. In the absence of CaM iNOS is highly unstable. The signalling cascade for Ca^{2+}-dependent eNOS/nNOS activation is initiated by ligand-induced activation of phospholipase C, which results in the formation of inositol (1,4,5)-trisphosphate, and the release of Ca^{2+} from inositol (1,4,5)-trisphosphate-sensitive intracellular Ca^{2+} stores. In the course of store depletion, store-operated Ca^{2+} channels are activated, resulting in a Ca^{2+} influx, referred to as capacitative Ca^{2+} entry. Both, release and influx of Ca^{2+} lead to a transient increase in $[Ca^{2+}]_i$. Ca^{2+} then binds to CaM and the Ca^{2+}/CaM complex associates with eNOS/nNOS leading to enzyme activation (Mayer, B. and Hemmens, B. 1997). By contrast iNOS is able to bind tightly to calmodulin even at very low

cellular concentration of calcium, and once synthetized it is active. The production of NO by iNOS is primarily controlled by transcriptional mechanisms. In most unstimulated cell types iNOS protein levels are either very low or undetectable. The most characteristic feature of iNOS is its prominent activation by proinflammatory cytokines (e.g. IFNγ and TNFα) or inhibition by antiinflammatory cytokines (e.g. IL-4, IL-10, IL-13 and transforming growth factor β (TGF$-\beta$). (Mac Micking J. et al (1997); Beck C. et al. (1999); Bogdan C. et al (2000).

1.3.2 Pathophysiological Roles of NO

Since the discovery that NO is able to induce vasodilation, a large number of other roles have been described for this messenger. NO has also been implicated in the control of vascular permeability and blood vessel formation (angiogenesis). It is also known to play a role in the immune system, the nervous system, in inflammation and in programmed cell death (Jaiswal M., et al. 2001).

1.3.3 Role in immune defense

NO can be produced by a number of cell types involved in local immune responses. In particular cytokine-activated macrophages can produce high concentrations of NO in order to kill target cells. NO-mediated cytotoxicity is often associated with the formation of nitrosyl thiol complexes in enzymes within the target cell. Killing by NO may also involve disruption of enzymes of the Kreb's cycle, reduced DNA synthesis, impaired mitochondrial function and induction of apoptosis. (Brüne et al. (1998); Bogdan C., et al, (2000))

1.3.3.1 Cellular responses mediated by NO

The effects of NO on cellular factors are generally classified as cGMP dependent or independent. Nitric oxide is able to activate cGMP signaling through the interaction of NO with the haem group of guanylate cyclase (Ignarro, 1990). The production of

cGMP leads to the activation of cGMP-dependent protein kinases and possibly to increased expression of cGMP-related genes (Beck C. et al. (1999); Pfeilschifter J. et al. (2001)). Another way by which NO activates cells is via protein kinase cascades. This was first reported by Lander et al. (1993). They report that, in human peripheral blood mononuclear cells, NO generating compounds stimulated a membrane-associated protein tyrosine phosphatase activity which led to a dephosphorylation and activation of the src family protein tyrosine kinase $p56^{lck}$ which is critically involved in T cell activation. Later it was shown that NO activates all three mitogen-activated protein kinase (MAPK) cascades in Jurkat T cells, i.e. the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) cascade, the stress-activated p38 MAPK cascade and the classical ERK/MAPK cascade (Lander et al., (1996); Pfeilschifter J. and Huwiler A. (1996); Huwiler A. and, Pfeilschifter J.(1999); Mühl H,et al. (1999)). Other possible targets for NO are represented the family of protein phosphatases. By direct interaction with this class of enzymes, either an activation or inhibition of phosphatase activity may be achieved, and this will lead to increased or decreased phosphorylation of their target proteins which include, importantly, members of the different MAPK pathways. Activation of a membrane-bound phosphatase was reported in T cells (Lander et al., (1993); for review see: Pfeilschifter et al. (2001)).

1.4 Transcription factors

The generation of the more than 200 different specialized cell types of the mammalian organism requires the establishment of diverse gene expression patterns that characterize the individual cell types.These patterns are formed through the combinatorial action of transcriptional regulatory proteins, some of which have the capacity to direct multipotent cells to assume a specific developmental fate. Transcription factors are proteins that bind to DNA near the start of transcription of a gene. These proteins either inhibit or assist RNA polymerase in initiation and maintenance of transcription.

1.4.1 NFκB

Nuclear factor kappa B (NF-κB) is a protein transcription factor that is required for maximal transcription of a wide array of proinflammatory molecules which are thought to be important in the generation of acute inflammation.
Members of the NF-κB family of transcription factors are expressed in most cell types of vertebrates. Five structurally related Rel/NF-κB proteins are known and can be detected as homo- and heterodimers (Baldwin, A. S., jr. 1996; Ghosh, S., et al. 1998). In nonstimulated cells NF-κB is sequestered in the cytoplasm in an inactive form via interaction with inhibitory proteins, the inhibitors of NF-κB (IκB) (Baeuerle P.A., and Baltimore D., 1988). The three most important IκBs are: IκBα IκBβ, and IκBε (Davis N., et al, 1991; Haskill S., et al, 1991; Thompson J.E., et al, 1995; Whiteside S.T., et al, 1997), bind the Rel homology domain (RHD) of NF-κB dimers und mask their nuclear localization signal (NLS)(Beg, A.A. et al. 1992; Ganchi, P.A., et al. 1992; Hatada, E.N.,et al. 1992;Zabel, U. et al.. 1993). Upon stimulation with various activators such as bacterial products, proinflammatory cytokines, viral proteins, and radiation, IκBs are rapidly phosphorylated at two serines located in their NH_2-terminal regulatory region. Thereafter, they are ubiquitinated, and degraded by a proteasome dependent mechanism (Nanterniet, and A.S. Baldwin, Jr. 1993; Henkel, T., et al. 1993). The exposure of the NLS results in a rapid nuclear translocation of NF-κB, which then binds decanucleotide sequences in responsive promotors. This process activates transcription of several genes. Among them are proinflammatory chemokines and cytokines, adhesion molecules, and antiapoptotic or cytoprotective genes (Ghosh, S., et al. 1998; Mayo, M.W., and A.S. Baldwin. 2000). IκB itself is a NF-κB inducible gene. After induction IκBα enters the nucleus, enhances NF-κB removal from DNA (Zabel, U., and P.A. Baeuerle. 1990), and takes NF-κB back to the cytoplasm, thus restoring the inducible cytoplasmic pool of the transcription factor (Arenzana-Seisdedos, F., et al 1995).
Lipopolysaccharide (LPS) stimulated recruitment of NF-κB to target genes in macrophages occurs in two temporally distinct phases. A subset of target genes whose promoter is already heavily acetylated before stimulation is immediately accessible to NF-κB and initiation of transcription occurrs fast. In contrast, other target genes are not immediately accessible to NF-κB.

The state of acetylation of DNA representing an ORF (open reading frame) is an indicator for its activity. Inactive DNA is wound up on histon-protein-comlexes which loose their affinity to DNA by acetylation. Acetylation of DNA actually means that the histon complexes get acetylated, loose their affinity to DNA and the DNA is transformed into its stretched form in which it can be transcribed more easily. Recruitment of NF-κB to sensitive promotors occurs within 120 min after nuclear entry and is preceeded by the formation of an initial transcription factor complex that directs the hyperacetylation of the promoter and makes it accessible to NF-κB. The dependency on modifications in chromatin structure for activation by NF-κB in certain genes is a mechanism allowing a highly selective regulation of these promotors in response to NF-κB (Simona S., et al, 2001).
In RAW macrophages and human peripheral blood mononuclear cells (PBMC) treatment with NO donors such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP) and GSNO resulted in enhanced binding activity of NF-κB family members (Lander et al., 1993; Knethen A., et al. 1999). In other cell types like in vascular endothelial cells activation of NF-κB binding following cytokine treatment can be inhibited by treatment with NO donors (Peng, et al, 1995). NO is a molecule with both anti-oxidant and pro-oxidant properties depending on the availability and concentration of potential reaction partners such as superoxide and hydrogen peroxide or other reactive oxygen species (ROS). This may explain the disparate observations in some cells (Pfeilschifter et al. 2001). Peroxinitrite (ONOO-) induces in a dose dependent manner the release of proinflammatory cytokines in human monocytes through activation of NF-κB DNA binding activity (Matata and Galinanes 2002).

1.4.2 AP-1

Activator protein-1 (AP-1) is an inducible transcription factor of the bZip family and is critical for the expression of many genes involved in the inflammatory response, including cytokines and adhesion molecules. Binding of TNF-α to its cell surface receptor activates AP-1 through a cascade of kinases that include a MAP kinase kinase kinase (MEKK1), a MAP kinase kinase (MKK4 and MKK7), and a MAP kinase [JNK (c-Jun N-terminal kinase)] (Yoshimura T., et al, 1987; Larsen C.G., et al, 1989).

AP-1 is composed of either Jun-Fos heterodimers or Jun-Jun homodimers (Smeal T., et al. 1989). AP1 binding sites can also interact with transcription factors of the CREB/ATF family by forming heterodimers with Jun and Fos proteins (Matthews J.R., et al, 1996; Rabbi M.F., et al.,1997). AP- 1 activity is controlled at both, transcriptional and posttranscriptional levels. Regulation at the posttranscriptional level is mediated by the state of protein phosphorylation. AP-1 transcriptional activity is dependent on the phosphorylation of two serine residues in the transactivation domain of Jun (Paine, R.D., etal., 1993). In addition AP1 binding activity is controlled by dephosphorylation of specific serine and threonine residues in the DNA binding domain of Jun (Standiford T.J., et al., 1991). In terms of transcriptional regulation, the c-*fos* promotor is rapidly activated (within 30min) in response to a variety of external signals, which lead to increased production of cFos protein and, when combined with a member of the Jun family, increased AP-1 biological activity (Karin, M., et al., 1997). AP-1 can be directly modificated by NO *in vitro*. The major components of AP-1 wich are c-Fos and c-Jun have single conserved cysteine residues within the DNA binding domains. These cysteine residues seem to be responsible for redox regulation of AP-1 activity. Nitrosylation of critical cysteines lead to the inhibition of AP-1 activity, suggesting that NO mediates regulation of the AP-1 transcription factor (Tabuchi et al., 1994).

1.4.3 Modulation of gene expression by NO

During the last years, an increasing number of genes have been shown to be under regulatory control by nitric oxide, including growth factors (Sasaki, et al, 1998; Tsurumi, et al., 1997; Chin, et al., 1997), cytokines (VanDervort, et al., 1994), chemokines (Villarete and Rernick, 1995; Mühl and Dinarello, 1997), receptors (Ichiki, et al., 1998) or enzymes (Hartsfield et al, 1997; Mühl and Pfeilschifter, 1995; for review see: Pfeilschifter et al. 2001). The gene for interleukin-8 (IL-8) has been show to be under transcriptional control by NO. In human endothelial cells, TNF-α-stimulated IL-8 production was inhibited by the NOS inhibitor (L)-N^G-nitroarginine methylester (L-NAME) in a dose-dependent manner. Moreover, exogenously added NO donors induced IL-8 expression (Villarete and Remick, 1995). Besides IL-8, TNF-α appears to be induced in a paracrine manner by endothelial and smooth-muscle-

derived NO in human neutrophils (VanDervort et al., 1994). Moreover, in human T lymphocytes NO mediated an increase in mRNA levels of TNF-α, which was ERK dependent (Deora A.A., et al, 2000). Furthermore, expression of macrophage inflammatory protein 1α (MIP-1α), another member of the chemokine family, was directly dependent on LPS-induced NO synthesis in PBMC (Mühl H. and Dinarello C., 1997), a process that may be initiated by PKB mediated activation of eNOS in human monocytic cells.

Vascular endothelial growth factor (VEGF), a growth factor mediating angiogenesis and vascular permeability, was found to be induced by NO in human glioblastoma and hepatocellular carcinoma cells. For both cell types, NO induced gene expression is mediated through the activation of, guanylate cyclase (Chin K. et al, 1997). In contrast, in mesangial cells NO-evoked expression of VEGF was independent of cGMP. Noteworthy, the regulation of the VEGF receptor Flt-1 was found to be regulated in NO/cGMP-dependent manner in mesangial cells (Frank et al.1999). Finally, NO is even able to trigger transcription of its own biosynthetic machinery: NO was found to increase IL-1β-induced iNOS gene expression at the transcriptional level in mesangial cells and vascular smooth muscle cells. Inhibition of NO synthesis clearly reduced IL1β-stimulated iNOS expression, suggesting that NO functions in a positive feedback loop that speeds up and strengthens its own biosynthesis (Mühl and Pfeilschifter, 1995; Boese M., et al.1996).

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acrylamide/bisacrylamide-solutions	Roth, Karlsruhe
Agar	Gibco Life Technologies, Eggenstein
Agarose	Biozym, Oldendorf
Ammoniumpersulfate	Sigma Biochemicals, Deisenhofen
Ampicillin	Sigma Biochemicals, Deisenhofen
Aprotinin	Roche Biochemicals, Mannheim
[γ-^{32}P]ATP	Amersham Pharmacia, Braunschweig
Bovine serum albumin	Sigma Biochemicals,Deisenhofen Gibco Life Technologies, Eggenstein
5-Bromo-4-chloro-3-indolyl-β-D-galactoside	Roth, Karlsruhe
Cell culture media	Gibco Life Technologies, Eggenstein
Complete (protease inhibitor)	Roche Biochemicals, Mannheim
[α-^{32}P]dCTP	Amersham Pharmacia, Braunschweig
DETA-NONOate	Alexis, Grünberg
Diethylpyrocarbonate	Sigma Biochemicals, Deisenhofen
Dithiothreitol	Sigma Biochemicals, Deisenhofen
Ethidium bromide	Sigma Biochemicals, Deisenhofen
Ficoll	Sigma Biochemicals, Deisenhofen
Fugene 6	Roche Biochemicals, Mannheim
Glutathione	Sigma Biochemicals, Deisenhofen
Guanidinium thiocyanate	Sigma Biochemicals, Deisenhofen
Hydroxylammoniumchloride	Merck, Darmstadt

8-Hydroxyquinoline	Sigma Biochemicals, Deisenhofen
IL-1β	Cell Concepts (Umkirch, Germany)
Isopropylthiogalactoside	Roth, Karlsruhe
Leupeptin	Roche Biochemicals, Mannheim
Lipopolysaccharide	Sigma Biochemicals, Deisenhofen
L-N^6-(1-iminoethyl)lysine	Alexis, Grünberg
β-Mercaptoethanol	Sigma Biochemicals, Deisenhofen
Molecular weight markers (DNA)	MBI Fermentas, St. Leon-Rot
Molecular weight markers (protein)	Amersham Pharmacia, Braunschweig
N^G-monomethyl-L-arginine	Alexis, Grünberg
N-naphtylethylendiamine dihydrochloride	Sigma Biochemicals, Deisenhofen
Nucleotide triphosphates	*PE* Biosystems, Weiterstadt
Oligonucleotides	Roth, Karlsruhe; Gibco Life Technologies, Eggenstein; MWG, Ebersberg
Pepstatin	Roche Biochemicals, Mannheim
Peptone	Gibco Life Technologies, Eggenstein
Phenol/Chloroform	Roth, Karlsruhe
Ponceau S	Sigma (Deisenhofen, Germany)
Polymyxin B (PmxB)	Sigma (Deisenhofen, Germany)
Pyrrolidine dithiocarbamate	Calbiochem-Novabiochem GmbH (Bad Soden, Germany)
RNasin	Promega, Mannheim
Rothi Load	Roth, Karlsruhe
Skim milk (non fat)	Fluka, Deisenhofen
Sodium nitroprusside	Sigma Biochemicals, Deisenhofen
Sodiumlaurylsarcosyl	Serva, Heidelberg
Triton X-100	Sigma Biochemicals, Deisenhofen
Tumor necrosis factor α (TNFα)	Knoll AG (Ludwigshafen, Germany)
tRNA, RNase free	Roche Biochemicals, Mannheim
Trypan blue	Gibco Life Technologies, Eggenstein
Tween 20	Sigma Biochemicals, Deisenhofen
[α-^{32}P]UTP	Amersham Pharmacia, Braunschweig

Yeast extract	Gibco Life Technologies, Eggenstein
Zeocin	InVitrogen, Groningen, Netherlands

Acetone, organic and inorganic acids, chloroform, ethanol, ether, methanol and isopropanol were from the supply store of the Universitätsklinikum Frankfurt. All other, not listed chemicals were purchased either from Merck (Darmstadt), or Roth (Karlsruhe) or Sigma Biochemicals (Deisenhofen).

2.1.1.1 S-nitroso-glutathione

Glutathione was dissolved in 0.625 N HCl on ice to a final concentration of 625 mM. $NaNO_2$ was added in an equimolar amount and the mixture was stirred at 0°C for 40 min. After the addition of 2.5 volumes of acetone stirring went on for additional 20 min, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone, and finally three times with diethylether and dried under vacuum. The powder was aliquoted in Eppendorf tubes and stored at –20°C until use.

2.1.2 Enzymes

Alkaline shrimps phosphatase	Roche Biochemicals, Mannheim
DNA polymerase T4	MBI-Fermentas, St. Leon-Rot
Pfu-DNA polymerase	Stratagene, Heidelberg
Turbo Pfu-DNA polymerase	Stratagene, Heidelberg
Proteinase K	Roche Biochemicals, Mannheim
Restriction enzymes	Roche Biochemicals, Mannheim; Stratagene, Heidelberg; MBI-Fermentas, St. Leon-Rot; NEB
Reverse transcriptase	Roche Biochemicals, Mannheim
RNA polymerase T3 and T7	Roche Biochemicals, Mannheim
RNase A and T1	Roche Biochemicals, Mannheim
T4-DNA ligase	Roche Biochemicals, Mannheim
Taq-DNA polymerase	*PE* Biosystems, Weiterstadt
TaqGold-DNA polymerase	*PE* Biosystems, Weiterstadt

2.1.2.1 Pretreatment of enzymes

2.1.2.1.1 Proteinase K

The lyophilized enzyme was dissolved in Aqua dest. (10 mg/ml), incubated for 30 min at 37°C and aliquoted. The aliquots were stored at –20°C until use.

2.1.2.1.2 RNase A

RNase A was dissolved to a final concentration of 10 mg/ml in RNase-buffer [Tris/HCl (10 mM, pH 7.5), NaCl (15 mM)]. The enzyme solution was incubated for 30 min at 95°C and cooled to room temperature over night. Aliquots were stored at –20°C until use.

2.1.3 Kits

Dnazol	Stratagene, Heidelberg
Cell Death Detedtion Elisa	Roche Biochemicals
ECL Detection Kit	Amersham Pharmacia, Freiburg
ECL Films	Amersham Pharmacia, Freiburg
Quick Spin Purification Kit	Machery &Nagel, Düren
Quick Change Site-Directed Mutagenesis Kit	Stratagene, Heidelberg
Promega Luciferase Assay System	Promega, Madison
Nanoquant	Roth, Karlsruhe
LDH- Assay	Boehriner Mannheim, Mannheim Germany
Rediprime Labelling Mix (RPN1633)	Amersham Pharmacia Biotech, Freiburg Germany
Midi-/Maxi-Kits for Plasmid purification	Quiagen, Hilden and Machery & Nagel, Düren both Germany
Optia Elisa Kits for IP-10 and IL-8 detection	Pharmingen
TOPO TA Cloning	Invitrogen, Groningen (Netherlands)
Trizol	Sigma Biochemicals, Deisenhofen

VEGF Elisa	R&D Systems, Nordenstadt (Germany)

2.1.4 Laboratory equipment

ABI-Prism 310 Genetic Analyser	Perkin Elmer Biosysterns, Weiterstadt
Gel dryer 583	Bio-Rad, München
GeneAmp 2400/9600 Thermocycler	Perkin Elmer Biosysterns, Weiterstadt
Gene Quant II	Amersham Pharmacia, Braunschweig
Herasafe clean bench	Heraeus, Hanau
Hyperprocessor	Amersham Pharmacia,
Incubator Heraeus BBD 6220	Heraeus, Hanau
Megafuge Haraeus 1.0 (rotor 7570F)	Heraeus, Hanau
Microplate reader Benchmark	Bio-Rad, München
Phospholmager BAS 1500	Raytest, Straubenhardt
Trans-Blot SD	Bio-Rad, München
TRI-CARB 2100 TR ß-counter	Canberra-Packard, Dreieich
Luminometer	Berthold

2.1.5 Antibodies and antisera

Anti-rabbit IgG (horseradish-peroxidase coupled)	Bio-Rad, Munich, Germany
Anti-human *HO-1* (rabbit, polyclonal)	Stressgene, Hamburg, Germany
Anti-human *iNOS* (mouse,monoclonal)	Pharmingen

2.1.6 Recombinant and purified proteins

IFN-γ (human)	PeproTech, Frankfurt
IFN-γ (murine)	Roche Biochemicals, Mannheim
IL-1β	Cell Concepts (Umkirch,Germany)
TNF-α	Knoll AG (Ludwigshafen, Germany)

2.1.7 Plasmids

2.1.7.1 Vectors

pBluescript II KS (+)	Stratagene, Heidelberg
pGL3-Basic vector luc	Promega, Mannheim
pCMV-galactosidase	Stratagene, Heidelberg
TOPO II	Stratagene, Heidelberg

2.1.7.2 Recombinant plasmids

pGL3 Luc(+) IL8 wild (Human,nucleotides -558- +98bp)	
pGL3 Luc(+) IL8 AP-1 (human,nucleotides -558- +98bp)	Vlahopoulos et al. 1999
pGL3 Luc(+) IL8 C/EBPβ (human,nucleotides -558- +98bp)	Vlahopoulos et al. 1999
pGL3 Luc(+) IL8 NF-κB (human,nucleotides -558- +98bp)	Vlahopoulos et al. 1999
pBKS(+)*GAPDHb* (human, nucleotides 148-302)	Tokunaga *et al.* 1987
pBKS(+)h*IL-8* (human, nucleotides 146-433)	Matsushima et al,1988
pBKS(+)*HO-1*	

(human, nucleotides 706-949)	
pBKS(+)*iNOS* (human)	kindly provided by Dr. H. Kleinert, Mainz
pBKS(+)*VEGF* (human, nucleotides 339-498)	Weindel *et al.* 1992
TOPO II(+)IP-10 (human, nucleotides 115-403)	
TOPO II(+)MIG (human, nucleotides 81-401)	

2.1.8 Bacterial strains

		Genotype
E.coli DH5α	Gibco Life Technologies, Eggenstein	*supE44 ΔlacU169 (∅80lacZΔM15) hsd R17 recA endA1 gyrA96 thi-1 relA1*
E.coli XL-1 blue	Stratagene, Heidelberg	*supE44 hsdR17 recA1 endA1 gyrA46thi relA1 lac- F`[proAB+ lacIq lacZΔM15 Tn10(tetr)]*
TOP10	Invitrogen, Groningen (Netherlands)	*F¯ mcrA Δ(mrr-hsdRMS-mcrBC)Φ80lacZ ΔM15 ΔlacX74 recA1 deoR araD139 Δ(araleu)7697 galU galK rpsL (Str^R) endA nupG*

2.1.9 Eukaryotic cell lines

DLD-1 cells Human colon carcinoma cells	Centre for Applied Microbiology & Research (Salisbury, United Kingdom)
CACO-2 cells Human colon carcinoma cells	German Collection of Microorganisms and Cell Cultures (Braunschweig,

	Germany)
U 937 cells Human promonocytic cell line	

2.1.10 Buffers

Deionizised Formamide	500 ml Formamide +50 g Mixed-bed resin (Bio-Rad 501-X8) Incubation at 4°C for 30 min, afterwards filtered, stored at -20°C
Denhardt Solution	1% (w/v) BSA 1% (w/v) Ficoll 400 1% (w/v) Polvinylpyrrolidon sterile filtered, stored at -20°C
FAB	80% (v/v) Formamide 1 mM EDTA 40 mM PIPES 400 mM NaCl
Fixingsolution (10 ml)	9,6 ml PBS 284 µl Formaldehyd 37% (1% endkonzentration) 100 µl Glutaraldehyd 25% (0,25% endkonzentration)
FLB 80	80% (v/v) Formamide 1 mM EDTA 1 x TBE 0,05% (w/v) Bromphenol blue 0,05% (w/v) Xylenecyanol
Hybridisation Buffer	250ml deionizesed formamide

(Northern Blot)	125 ml SSC
	50 ml 50x Denhardt Solution
	50 ml 10% SDS
	20 ml H_2O
	50 g Dextransulfat
	stored at -20°C
Hybridisation Buffer (3x)	12% (w/v) Ficoll
(EMSA)	60 mM Hepes ph7,9
	150 mM KCl
	3 mM EDTA
	3 mM DTT
	3 mM PMSF
	0,75 mg/ml BSA
	stored at -20°C
Laemmli-buffer (4 x)	125 mM Tris/HCI pH 6,8
	10% (w/v) SDS
	50 mM Dithiothreitol
	30% (v/v) Glycerol
	0,01% (w/v) Bromphenol blue
LB	I% (w/v) Trypton
	0,5% (w/v) Yeast extract
	1% (w/v) NaCl
LB-agar	1 % (w/v) Trypton
	0,5% (w/v) Yeast extract
	1% (w/v) NaCl
	1,5% (w/v) Agar
Lysis-buffer	300 mM NaCl
	50 mM TrisHCl pH 7,6

	0.5% Triton X-100 supplemented with protease inhibitor cocktail
10xMOPS (ph7,2):	0,4M MOPS 0,1 M Na-acetat 0,01 M EDTA
10xPAGE	250 mM Tris 1 % (w/v) SDS 520 mM Glycin
10 x PBS (pH 7,4)	1,3 M NaCl 30 mM NaH_2P0_4 70 mM Na_2HP0_4
RNase buffer	10 mM TRIS/HCl pH 7,5 0,3 M NaOAc pH7,0 5 mM EDTA
SOB –Medium	2% (w/v) Bacto Trypton 0,5% (w/v) Yeast Extract 10 mM NaCl 2,5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$
SOC –Medium	2% (w/v) Bacto Trypton 0,5% (w/v) Yeast Extract 10 mM NaCl 2,5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glucose

STET	8% (w/v) Saccharose 50 mM Tris/HCl pH 8,0 50 mM EDTA pH 8,0 0,1% (v/v) Triton X-100
20 x SSC (pH 7.0)	3 M NaCl 0,3 M Sodium citrate pH 7,0
50 x TAE	2 M Tris 1 M Acetic acid 50 mM EDTA
TB – Buffer(ph 6,7)	10 mM Pipes 15 mM $CaCl_2$ 250 mM KCl 55 mM $MnCl_2$
100 x Mg^{2+}-buffer	100 mM MgCl2 5 mM 2- mercaptoethanol
0.1 M NaP04-buffer	100 ml of 0.1 M Na_2HPO_4 adjusted to pH 7,3 at 37°C using 0.1 M NaH_2P0_4
ONPG-solution	4 mg/ml in OJM $NaP0_4$ buffer
Stainingsolution (10ml)	8,75 ml PBS

	1 ml Ferro-Ferri (Endkonzentration 10%) 250 µl X-Gal-Lösung (Endkonzentration 2,5%) 25 µl $MgCl_2$ (1M, Endkonzentration 0,25%)
Stop solution	1 M Na_2CO_3 solution
10 xTBE	0,45 M Tris 0,45 M Boric acid 10 M EDTA
50 x TBST	0,1 M Tris 1,5 M NaCl 5% (V/V) Tween 20
Transfer buffer (Western blot)	25 mM Tris 192 mM Glycine pH 8,3 20% (v/v) Methanol
Puffer A (cytosolic extract):	10 mM Hepes (pH 7,5) 10 mM KCl 0,1 mM EDTA 0,1 mM EGTA Complete protease inhibitor (freshly added)
Puffer B (nuclear extract):	20 mM Hepes (pH 7,5) 0,4M NaCl 1 mM EDTA 1 mM EGTA 1 mM DTT Complete protease inhibitor (freshly added)

All buffers were prepared with highly purified water from a Milli-Q-system (Millipore).

2.1.11 Buffers, media and sera for cell culture

Dulbecco's modified Eagles medium	Gibco, Berlin
Phosphate buffered saline	Gibco, Berlin
Fetal calf serum	Gibco, Berlin
Penicillin/Streptomycin	Gibco, Berlin
Trypsin/EDTA	Gibco, Berlin

2.1.12 Computer Software

DNA/protein homology search	BLAST search (National Center of Biotechnology, USA; URL: *http://www.ncbi.nlm.nih.gov*)
Graphic processing	Corel Draw 8.0/10
Presentations	Powerpoint 2000
Statistical analysis	Sigma Plot 4.0/5.0
Text processing	Microsoft Word 2000
Vector design	Clone Manager 5.02

Programs belonging to special devices are mentioned separately in the appropriate sections.

2.1.13 Statistics

For experiments using DLD-1and U937 cells data are shown as means ± SD. For experiments with PBMC data are shown as means ± SEM. Data are presented either as pg/ml, as ng/ml, or as fold-induction compared to unstimulated control and were analyzed by unpaired Student´s t test (DLD-1,U937 cell experiments) or paired Student's t test (PBMC experiments) on raw data using Sigma Plot (Jandel Scientific).

2.2 Methods

2.2.1 Cell culture

All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 (Heraeus BBD 6220 incubator). For subcultivation, cells were washed once in phosphate-buffered saline (PBS), subsequently trypsinized (Trypsin, EDTA) and diluted in a 1:5-20 ratio. For long-term storage, the cells were treated as follows: after trypsinization, cells were concentrated (5 min at 1100 rpm, Heraeus Megafuge 1.0, rotor 75750F), washed with PBS, concentrated as above, diluted in freezing medium (growth medium supplemented with 10% DMSO) and stored in cryotubes (Nunc). The cryotubes were cooled down slowly (1°C per minute to -80°C) and finally stored in liquid nitrogen .

2.2.1.1 Cultivation of DLD-1 and Caco-2 colon carcinoma cells

Human DLD-1, Caco-2 colon carcinoma/epithelial and U937 cells were obtained from the Centre for Applied Microbiology & Research (Salisbury, United Kingdom) and the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The colon carcinoma cells were maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (GIBCO BRL, Eggenstein, Germany). For the experiments, confluent cells on polystyrene plates (Greiner, Frickenhausen, Germany) were washed with PBS and incubated in the aforementioned medium. Cells were then incubated for varying periods on fresh DMEM containing the factors or reagents of interest.

2.2.1.2 Cultivation of U937 cells

The human macrophage cell line U937 was maintained in RPMI1640 buffered with 25 mM hepes (Gibco Life Technologies) containing 10% FCS (Gibco Life Technologies), 2 mM L-glutamate, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco Life Technologies). Before each stimulation the medium was changed. To pellet the cells (U937 cells are nonadherend cells) they were centrifuged for 5 min

at 1000 rpm (Heraeus Megafuge 1.0, rotor 75750F). Cells were then incubated for varying periods on fresh RPMI containing the factors or reagents of interest.

2.2.1.3 Isolation, cultivation and stimulation of peripheral blood mononuclear cells (PBMC)

The study protocol and consent documents were approved by the *Ethik Kommission* of the Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main. Healthy volunteers abstained from any drugs during 2 weeks before the study. Blood was drawn into heparinized syringes (10 U/ml final concentration) and PBMC were isolated by centrifugation through Ficoll (Histopaque-1077, Sigma Biochemicals) for 15 min at 2000 rpm (Heraeus Megafuge 1.0, rotor 75750F). Cells obtained by this procedure were then washed three times with PBS. PBMC were resuspended in RPMI 1640 supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco Life Technologies) and 1% (v/v) heat-inactivated human serum (Sigma Biochemicals) and seeded at 3 x 10^6 cells/ml in round-bottom polypropylene tubes.

2.2.1.4 Measurement of cell parameters

2.2.1.4.1 Cell viability

At the experimental time points of interest, cells were trypsinized (1x Trypsin/EDTA) and diluted in fresh DMEM. One part of the cell suspension was mixed with one part of 0.4% trypan blue (Gibco Life Technologies) and incubated for 3 min at room temperature. Subsequently, unstained (viable) and stained (non-viable) cells were counted using a Neubauer chamber.

2.2.1.4.2 Apoptosis

2.2.1.4.2.1 Detection of cell death by analysis of cytosolic oligosome-bound DNA and release of lactate dehydrogenase (LDH) activity

Apoptotic cell death is characterized by membrane blebbing, condensation of cytoplasm and the activation of endonucleases which cleave double stranded DNA at the most accessible internucleosomal linker regions, finally generating mono- and oligonucleosomes. As DNA degradation occurs several hours before plasma membrane disassembly, these mono- and oligonucleosomes are enriched in the cytoplasm of apoptotic cells. The ELISA determins soluble DNA/histone complexes as a readout for cellular rates of apoptotis (Cell Death Detection ELISA, Roche Biochemicals). Cytosolic oligonucleosome-bound DNA is quantified. Absorbance values (OD 405/495 nm) give a relative measure of ongoing DNA fragmentation, a common marker for cell death by apoptosis.
Release of LDH which is normally only intracellular in a high concenration is also an indicator for ongoing apoptosis that was determined using an assay-kit according to the manufacturer´s instructions (Boehringer Mannheim).

2.2.1.4.3 Nitric oxide synthase activity, as determined by the amounts of nitrite produced using the Griess reagent

Nitrite, a stable NO oxidation product, is determined with the Griess reaction (Green *et al*. 1982) and used as a direct readout for nitric oxide synthase activity. Cell culture supernatants were cleared by centrifugation (5 min at 400 g). 100 µl of the cleared lysates or supernatants were mixed with 100 µl Griess reagent (Merck). After 5 min at room temperature, the absorbance was measured at 540 nm with a reference wavelength at 595 nm.

2.2.2 Bacterial culture

The *E. coli* strains DH5α (Gibco Life Technologies) and XL1-blue (Stratagene) were used for amplification of plasmid DNA. Both strains were either grown in liquid LB (Lauria-Bertani) or SOC medium. For selection, the media contained ampicillin (50

µg/ml). Agar-plates were generated with LB-ampicillin medium supplemented with agar (15 g/l). For long-term preservation of transformed bacteria (*2.5.2*), cells were mixed with sterile glycerol [30% (v/v)] and stored at –80°C.

2.2.2.1 Competent bacteria for transformation

To yield high transformation efficiencies from plasmid DNA in bacteria, cells have to be pretreated chemically. To this end, 250 ml SOB-Medium were inoculated with 200 µl of an overnight bacterial culture and grown at 18°C until the suspension reaches an optical density of 0.5 ($OD_{600\ nm}$). The bacterial growth was stopped by storing the suspension for 10 min on ice. Bacterial cells were concentrated by centrifugation (15 min, 2500g, 4°C; Heraeus Megafuge 1.0, rotor 7570F). The cellular pellet was subsequently resuspended in 80 ml solution TB, mixed and incubated on ice for 10min. The bacterial suspension was centrifuged again (15 min, 2500 g, 4°C), the pellet was gently resuspended in 20 ml of solution TB+7%DMSO and incubated on ice for additional 10 min. Aliquots of competent bacteria were snap frozen in liquid nitrogen and stored at –80°C.

2.2.2.1.1 Transformation

100 µl of a competent bacteria suspension (2.2.2.1) was thawed on ice and 1-5 µl of the ligation reaction containing recombinand plasmids (see 2.2.3.7.2) were added. The bacteria/DNA mixture remained on ice for additional 30 min followed by an incubation at 42°C for 30 s. The bacteria were chilled on ice again for 2 min, before 400 µl of SOC-medium was added. For initial expression of plasmids encoding ampicillin resistance, bacteria were incubated for 45 min at 37°C on a shaker. Subsequently, 100 µl of this transformation culture was plated on ampicillin containing agar plates. To enable a blue/white screening for recombinant clones, the agar plate was supplemented with 50 µl X-Gal (2% in DMF) and 50 µl IPTG (0.1 M in $Aqua_{dest}$). The plates were incubated overnight at 37°C.

2.2.3 Nucleic acid techniques

2.2.3.1 Preparation of plasmid DNA

Plasmids have been routinely isolated from bacteria cultures using a modified protocol originally described by Birnboim and Doly (1979). 3 ml of medium containing the appropriate antibiotic(s) were inoculated with a single bacterial colony from a selective agar plate and incubated overnight by vigorous shaking at 37°C. 1.5 ml of the cell-suspension was centrifuged for 2 min at 7000 g, and the medium was removed carefully by aspiration. The bacterial pellet was resuspended in 100 µl of solution I. Subsequently, 200 µl of freshly prepared solution II were added to the dispersed bacteria, mixed five times by inverting the tubes and stored on ice for exactly five minutes. This step lyses the bacterial cells and denatures the DNA. The lysate was neutralized by 150 µl acidic potassium buffer (solution III), which was gently mixed after addition and stored on ice for additional 10 minutes. The high salt concentration causes SDS to precipitate, and the denatured proteins, cellular debris and chromosomal DNA become trapped by salt-detergent complexes. Plasmid DNA, being smaller and covalently closed, renatures correctly and remains in solution. The tube was centrifuged for 10 min at 15,000 g and the supernatant containing the plasmids transferred to a fresh Eppendorf tube. A subsequent cleaning step using phenol/chloroform was performed optionally, as trace amount of phenol could disturb subsequent enzymatic reactions processing the plasmid-DNA. Plasmid-DNA was precipitated using 2 volumes of ethanol at room temperature and a centrifugation step for 10 min at 15,000 g. The pellet was air dried for 10-15 min and the DNA was finally dissolved in 15 µl of TE-RNAse. High amounts of pure plasmid DNA (up to 100 µg) were prepared using the QIAGEN Plasmid Midi Kit or for transfections the QIAGEN ENDOFREE Plasmid Maxi Kit as described by the manufacturer.

Solution components	Solution I	Solution II	Solution III
Glucose	50 mM	---	---
Tris/HCl (pH 8.0)	25 mM	---	---
EDTA (pH 8.0)	10 mM	---	---
NaOH	---	200 mM	---
SDS	---	1%	---
KOAc (pH 5.5, adjusted with glacial acetic acid)	---	---	3 M

2.2.3.2 RNA isolation from cultured cells

RNA isolation was performed with TRI-Reagent according to the protocol from the manufactor (Sigma Chemicals, Deisenhofen). Cells were grown and stimulated as described above (2.2.1). Last traces of media were removed by a pipette tip attached to a vacuum line. Subsequently, cells were lysed with 1ml of TRI-Reagent per 10 cm-plate, the lysate was transferred into an Eppendorf tube. After addition 200 µl chloroform, the samples were inverted five times. The inverted tubes were incubated at room temperature for 5 min, centrifuged (15,000 g, 15 min) and the aqueous upper phase was transferred into a fresh tube. RNA was precipitated using 500 µl of Isopropanol, and isolated by a single centrifugation step (15,000 g, 15 min). The RNA pellet was washed in cold DEPC-treated water/70% Ethanol, followed by a centrifugation (15,000 g, 10 min). The final RNA pellet was resuspended in 50 µl DEPC-treated water. Following a 10 min incubation at 55°C, the amount of isolated RNA was quantified photometrically (2.2.3.3). 3 µg of the isolated RNA was controlled for integrity by agarose gel electrophoresis (1%, 1x TBE buffer). Finally, RNA was stored at –20°C until use.

2.2.3.3 Quantification of nucleic acid concentrations

Concentrations of nucleic acids were determined photometrically using a wavelength of 260 nm (Gene Quant II, Amersham Pharmacia). An optical density (OD) of 1 corresponds to approximately 50 µg/ml double-stranded DNA or 40 µg/ml for single stranded DNA and RNA (Sambrook *et al*. 1989). The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimation of the purity of the nucleic acid preparation. Highly pure DNA or RNA are characterized by ratios between 1.8 and 2.0.
The concentrations were calculated according to the following equation:
$C[\mu g/ml] = OD_{260} \times V \times F$
V = dilution factor
F = multipication factor (dsDNA = 50; RNA = 40)

Low amounts of DNA were estimated by agarose gel electrophoresis (2.2.3.4) in comparison with a known standard concentration.

2.2.3.4 Agarose gel electrophoresis of nucleic acids

Nucleic acids were usually separated by gel electrophoresis using agarose gels. The gel strength was dependent on the molecular weight of the analyzed nucleic acids. Agarose (Roth/Gibco Life Siences) was dissolved in 1x TBE gel electrophoresis buffer. Ethidium bromide was added to a final concentration of 500 ng/µl. Ethidium bromide binds to DNA or RNA by intercalation between the bases and, thus enables an ultraviolet fluorescence illumination of nucleic acids. The DNA/RNA probes were diluted with loading buffer [6x loading buffer: 30% glycerol (v/v), 0.25% bromophenol blue (w/v), 0.25% xylenecyanole (w/v), 60% 10x TBE buffer (v/v)] and transferred into the appropriate gel wells. Electrophoresis was performed in 1x TBE buffer with a voltage of 5-10 V/cm gel. DNA fragment sizes were estimated using molecular weight markers (MBI Fermentas).
For separation of DNA molecules from 0,5 to 7 kbp normally 1%age ([w/v] Agarose) gels were used. Smaller DNA fragmentes (500bp -100bp) were separated in gels with higher densities (mostly 2%) (Sambrook et al., 1989).

2.2.3.5 DNA isolation from agarose gels

The use of the Quick Spin Purification Kit (Machery & Nagel) enables a pure extraction of DNA fragments directly from agarose gels. The system is based on a silica matrix, which binds single and double stranded DNA. The DNA fragments of interest were cut from the gel with a razor blade and further processed according to the instructions of the manufacturer.

2.2.3.6 Reverse transcriptase polymerase chain reaction

2.2.3.6.1 Reverse transcription

The enzyme reverse transcriptase, originally discovered in RNA tumor viruses, synthesizes a complementary DNA strand using RNA as a template. This enzymatic activity provides access to the generation of cDNA. Random hexamers (50 ng/µl) were used as internal enzyme start sites.

Reverse transcriptase reaction:	X µl (*max. 3 µl)*	RNA (1 µg)
	4 µl	$MgCl_2$ (25 mM)
	2 µl	10x PCR-Buffer
	2 µl	d**A**TP (each 10 mM)
		d**C**TP
		d**G**TP
		d**T**TP
	1 µl	RNase Inhibitor (20U/µl)
	1 µl	Random Hexameric Primers (50 µM)
	1 µl	Reverse Transcriptase
		(50U/µl; Muloney Virus)
		Applied Biosystems Applera
	X µl	DEPC H_2O
	20 µl	**Total Volume**

1 µg of total RNA was added to the reagents as listed in the table. The reaction was performed for 30 min at 42°C. Subsequently the mix was terminated by incubation for 5 min at 99°C and cooled to 4°C. Aliquots of the cDNAs were stored at 4°C.

2.2.3.6.2 Polymerase chain reaction (PCR)

This methods enables *in vitro* amplification of DNA fragments without time consuming cloning and identification steps (Mullis & Foloona, 1987). The method is based on the availability of heat-stable DNA polymerases which allow multiple denaturing of template DNA, annealing of driver sequences (primer) and synthesis of DNA by amplification steps within one tube.

Polymerase chain reaction:		
	2 µl	RT-product (2.2.3.6.1) or
	X µl	Plasmid-DNA Template (5-10 ng) or
	X µl	genomic-DNA Template (250 ng)
	5 µl	10x PCR-Buffer
	3 µl	$MgCl_2$ (25 mM)
	1 µl	d**A**TP (each 10 mM)
		d**C**TP
		d**G**TP
		d**T**TP
	5 µl	Forward/Reverse Primer-Mix (5 µM)
	0.4 µl	*Taq-Gold* Polymerase (5U/µl)
	X µl	DEPC H_2O
	50 µl	**Total Volume**

The reaction was performed in a thermocycler (GeneAmp 2400 or 9600, *PE* Biosystems). Conditions varied depending on the gene of interest, the type of polymerase used for amplification and the experimental set up. For **semi-quantitative gene analysis** *taq-gold* polymerase was used. This enzyme has to be activated by a single incubation for 10 min at 94°C (hot start!). In general, 25-30 cycles of the following steps have been carried out: 1 min 94°C, 1 min 60°C (primer-annealing) and 2 min 72°C (elongation). The amplification was completed by a final 7 min incubation step at 72°C. Depending on special conditions of template, primers and type of cell the protocol was adapted individually (see table below). The samples were stored at 4°C and analyzed by gel electrophoresis.

Semi-quantitative gene analysis for	Annealing temperature	Cycle number
GAPDH	60°C	23-25
hiNOS	62°C	30-32
IL8	60°C	28
IP10	60°C	30

For **amplification of genomic DNA** *taq* and *(turbo)-pfu*-polymerase has been used. In contrast to *taq* polymerase the *(turbo)-pfu* polymerase provides proofreading activity, at a reduced rate of synthesis. 30–35 cycles were sufficient to amplificate genomic IL-8 promotor fragments by using to following PCR program: 1 min 94°C, 1 min 60°C and 2 min 72°C. Finally, the amplification was completed by a 7 min incubation step at 72°C.

2.2.3.7 Cloning of PCR products

After amplification, RT-PCR products were cloned into the pBluescript II (+) KS vector (Stratagene) or the TOPOII vector (Invitrogen). To this end, the DNA fragments were separated in a agarose gel, cut out and isolated with the Quick Spin Purification KIT (2.2.3.5). The DNA fragment of interest was reamplified by *pfu*-polymerase (Stratagene) (not in case of cloning into the TOPOII vector), purified by gel electrophoresis and ligated into the pBluescript II (+) KS vector at the *Eco*RV restriction site as described in 2.2.3.7.2 or ligated into the TOPOII vector at the multiple cloning site according to the instruction manual of Invitrogen.

2.2.3.7 Manipulation of DNA

2.2.3.7.1 Restriction

Type II endonucleases isolated from bacteria specifically bind palindromic sequences with a subsequent cleavage of the DNA molecule at their recognition site. This process generates either blunt-end fragments or overhanging cohesive ends, which allow the generation of recombinant DNA by enzymatic ligation. The

standard approach for DNA digestion is subsequently listed. After incubation at the appropriate temperature, DNA cleavage was checked by agarose gel electrophoresis (2.2.3.4).

DNA digestion:	17.8 µl	DNA (500 ng)
	2.0 µl	10x buffer
	0.2 µl	restriction enzyme (3 units)

2.2.3.7.2 Ligation

Generation of covalent phosphodiester bonds between the 5´-phosphate and the 3´-OH of DNA fragments is catalyzed by T4-DNA ligase. The ligation reaction was performed with restricted or PCR amplified DNA. The desired DNA fragments were directly purified with a Quick Spin Colum (Machery& Nagel) or after separation by agarose gel electrophoresis. The mixture was incubated for up to 16h at room temperature. Thereafter, an aliquot of this reaction was transformed into competent bacteria as described in section 2.2.2.1.1.

DNA ligation:	4 µl	10x ligase buffer
	1 µl	vector DNA (500 ng)
	10 µl	DNA fragment
	1 µl	T4-DNA ligase (1 unit)
	ad 40 µl	H_2O

2.2.3.7.3 Dephosphorylation

If the vector DNA carried complementary cohesive termini after enzymatic restriction, the plasmid was treated with alkaline phosphatase (Roche Biochemicals) prior to ligation. Phosphatases remove the 5´-phosphates from DNA strands. This technique prevents a religation of restricted plasmids without

incorporation of insert DNA. To this end, the restricted DNA was incubated with alkaline phosphatase (0,1 unit/µg DNA) for 30 min at 37°C. Afterwards, the phosphatase was heat-inactivated for 10 min at 70°C and the denatured protein extracted with phenol/chloroform. The ligation of the DNA fragments was performed as described in *2.6.6.2*.

2.2.3.7.4 DNA sequencing

DNA sequencing was performed using the ABI-Prism 310 Genetic Analyser (*PE* Biosystems) based on the dideoxynucleotide chain termination method (Sanger *et al.* 1977). In the termination labeling mix, the four dideoxy terminators (ddNTPs) are tagged with different fluorescent dyes. This technique allows the simultaneous sequencing of all four reactions (A, C, G, T) in one reaction tube. The probes were separated electrophoretically using a micro capillary. As each dye terminator emits light at a different wavelength when excited by laser light, all four colors corresponding to the four nucleotides can be detected and distinguished within a single run. Raw data were evaluated by the Abi Prism sequencing analysis software on a Power G3 Macintosh computer. The sequencing reaction, as listed in the table, was performed in a thermocycler (GeneAmp 2400, *PE* Biosystems) with 25 cycles of the following temperature steps: 96°C for 10 sec, 55°C for 5 sec, 60°C for 2 min. For the detection process, samples were prepared as described by the manufacturer.

DNA sequencing:	1 µl	DNA (250 ng plasmid DNA or 50 ng PCR derived DNA)
	2 µl	sequencing premix
	1 µl	primer (5 pmol)
	6 µl	H_2O

2.2.3.8 RNase protection assay

This method was used to quantify the amounts of specific RNA transcripts from total cellular RNA. Compared to Northern blot analysis, this technique possesses increased sensitivity and specificity. The assay is based on the principle, that double stranded RNA hybrids were protected from cleavage by RNases A and T1.

2.2.3.8.1 DEPC-treatment

Buffers and solutions for RNA based methods were treated with diethyl pyrocarbonate (DEPC) to inactivate RNases. Note that DEPC reacts with amines such as Tris. Hence, buffers containing amines were made from DEPC treated water, but not treated with DEPC directly. DEPC treated water was obtained by adding 1 ml DEPC per liter of Aq. dest. After mixing in an overnight step at RT, the solution was autoclaved.

2.2.3.8.2 Preparation of a radiolabeled antisense probe

The desired probes (150-450 nt in length) were cloned into the transcription vector pBluescript II KS (+) as described in section 2.2.3.6.3. The recombinant plasmids were linearized with restriction enzymes, phenol/chloroform extracted, precipitated and dissolved in $Aqua_{dest}$ to a final concentration of 1 µg/µl. A pure single stranded, [α-^{32}P]UTP radiolabeled antisense transcript was synthesized using T3 or T7 RNA polymerase. The reaction mix was prepared as listed in the table and incubated for 1 h at 37°C.

In vitro transcription:	1.1 µl	H_2O, DEPC-treated
	1.0 µl	nucleotides (ATP, CTP, GTP; each 5 mM)
	2.0 µl	transcription buffer (5x)
	0.4 µl	RNasin (40 U/µl)
	1.0 µl	T3 or T7 RNA polymerase, depending on the vector

2.5 µl [α-^{32}P]UTP (800 Ci/mM)
1.0 µl Linearized template (1 µg/µl)

Subsequently 90 µl DEPC-H_2O were added. The reaction was extracted with an equal volume of phenol/chloroform and centrifuged (15,000 g, 2 min). The radiolabeled RNA was precipitated form the aqueous phase using 40 µl of 7,5 M ammoniumacetate, 1,5 µl of tRNA (10 µg/µl) and 350 µl of ethanol for 15 min at –20°C. Following a 15 min centrifugation step at 4°C, the radiolabeled pellet was dissolved in 20 µl FLB 80 and purified using an acrylamide/urea gel (5% acrylamide/bisacrylamide, 29:1; 8 M urea; 1x TBE). Electrophoresis (1x TBE buffer, 300 V) was terminated after 90 min. The radiolabeled probe was located by autoradiography, cut out and eluated into 300 µl elution buffer (0,1x TBE, 0,2% SDS). The incorporated activity of the freshly prepared radiolabeled probe was determined using a β-counting device (TRI-CARB 2100 TR, Canberra-Packard).

2.2.3.8.3 Hybridization and cleavage

10-30 µg of total RNA from cell culture experiments (see 2.2.3.2) were used for the experiments. Total RNA and 100,000 cpm of the radiolabeled probe were co-precipitated using a 2,5-fold excess of ethanol/0,1 M NaOAc. The samples were incubated at –20°C for 15 min and subsequently centrifuged (15,000 g, 10 min). The supernatants were removed and the RNA/antisense probe pellet was resuspended carefully in 30 µl FAB hybridization buffer. Samples were subsequently denatured for 10 min at 85°C and hybridized overnight (42°C in a water bath). Thereafter, samples were treated with 300 µl RNase T1/A-mix (RNase buffer supplemented with 10 µg of RNase A and 200 units of RNase T1) for 1 h at 30°C (Melton *et al*. 1984)]. Under these conditions, every single mismatch was recognized by the RNases. RNases were inactivated by an addition of 6,6 µl SDS (10%) and 4,4 µl proteinase K (10 µg/ml) for 15 min at 42°C. The samples were extracted with phenol/chloroform and centrifuged (15,000 g, 2 min). Protected double-stranded RNA hybrids were precipitated using 880 µl of ethanol and 1,5 µl of tRNA (10 µg/µl). After centrifugation, (15,000 g, 15 min), the pellet was

resuspended in 25 µl FLB 80 buffer, heated for 5 min at 95°C and loaded on the gel.

2.2.3.8.4 Analytical gel electrophoresis and signal detection

The protected RNA fragments were separated on acrylamide/urea gels (5% acrylamide/bisacrylamide, 29:1; 8 M urea; 1x TBE). 1000 cpm of the radiolabeled antisense probe served as a size marker. Electrophoresis (300 V, 1x TBE buffer) was stopped after 60 min. The gel was fixed on Whatman 3MM paper and subsequently dried on a gel drying system (Bio Rad). The radiolabeled gel was exposed to a phosphoimager plate (BAS-MP 2040S, Fuji) and analyzed using a Fuji PhosphoImager BAS-1500.

2.2.3.9 Northern blot analysis

This method is used to quantify the amounts of specific RNA transcripts from total cellular RNA. Compared to RNAse Protection Assays, this technique has the advantage to be less a source of error. By stripping the membranes (2.2.3.9.3) multiple genes can be investigated using the same blot. The assay is based on the principle, that immobilized RNA strands on a membrane could specificly hybridize to radioactive labeled DNA single strands.

2.2.3.9.1 Generation of Probes

The cDNA inserts of human IP-10 and MIG were generated by reverse transcription (RT) from mRNA of DLD-1 cells stimulated with IL-1β and IFNγ. The following primers were used for MIG: 5'-GCT GGT TCT GAT TGG AGT GC-3' (for) and 5'-TGA CGA GAA CGT TGA GAT TTT C-3' (rev) for IP-10: 5'-AGT GGC ATT CAA GGA GTA CC-3' (for) and 5'-ATC CTT GGA AGC ACT GCA TC-3' (rev). The sticky ended fragments were cloned into the multiple cloning site of the Top-II-Vector (Clontech, Germany). The cDNA fragments were exised by a EcoRI digestion.

2.2.3.9.2 Preparation of a radiolabeled antisense probe

The desired probes (300-1000nt in length) were cloned into the transcription vector TOPO II (+) as described in section 2.2.3.6.3. The fragments of interest were cleaved out of the recombinant plasmids by use of the restriction enzyme Eco RI (MBI Fermentas, Litauen), gel isolated, extracted with Quick Spin Tubes and brought to a final concentration of 10 ng/µl. A pure single stranded, [γ-^{32}P]dCTP radiolabeled antisense transcript was synthesized using the Rediprime Labelling Mix (Amersham RPN1633) according to the manufactor's protocol. The labeled probe was cleaned up by using Nick Colums (Amersham, Pharmacia) according to the manufactor's protocol.

2.2.3.9.3 Analytical gel electrophoresis and signal detection

Total cellular RNA was extracted from DLD-1 cells using the TRI-Reagent as described above. Samples of 10 µg of RNA were separated on 1.4% agarose/formaldehyde gels and transferred by sandwich blot to a gene screen membrane. Membranes used for RNA transfer were obtained from NEN Life Science Products (Boston, MA, USA).
After UV crosslinking with 150 mJ the membrane was stained with Methylenblue to check if the transfer of RNA was successfull and the loading of the gel was equal. The membrane was destained by incubation first with 20% ethanol then with 0,2 x SSC/1%SDS. After prehybridization for 2h the filters were hybridized for 16h at 42°C to a ^{32}P-labeled cDNA insert of interest. DNA probes were radioactively labeled with ^{32}P-dCTP by random priming (Amersham Pharmacia Biotech, Freiburg, Germany). Finally, the filters were washed twice with 2 x SSC/ 0,1% SDS for 2 x 20 min at 42°C and several times at 65°C with 0.2 x SSC/1% SDS. To correct for variations in RNA amounts the blot was stripped. Thereafter blots were finally rehybridized with a ^{32}P-labeled insert from a human ß-actin cDNA clone. Specific signals were detected using, an automated detector system BAS 1500 from Fujifilm (Raytest, Straubenhardt, Germany).

2.2.3.10 Protein techniques

2.2.3.10.1 Preparation of lysates

2.2.3.10.1.1 Cell lysates

Cells were grown and stimulated as described above (2.2.1). For harvesting, cells were washed twice with ice-cold PBS. Last traces of PBS were removed by a pipette tip attached to a vacuum line. DLD-1 cells were treated with lysis-buffer (300 mM NaCl, 50 mM TrisHCl, pH 7.6, 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). To remove cellular debris, samples were centrifuged (15,000 g, 10 min, 4°C) and the supernatants were stored at –80°C until use.
Protein concentrations were determined using the Roth Nanoquant Protein Assay (2.2.3.10.1.2).

2.2.3.10.1.2 Determination of protein concentration

The amount of protein in cellular lysates was determined using the Roth Nanoquant Protein Assay (Bradford method). 50 µl of the samples (1:5-1:50 prediluted in homogenization buffer) were pipetted in duplicate into appropriate wells of a 96-well ELISA plate. Different BSA concentrations (20-100 µg/ml) were used as a standard. 200 µl of Roth solution (1:5 diluted in $Aqua_{dest}$) were added to each well. After 10 min of incubation, the optical density was measured at a wavelength of 595 nm and reference wavelength 450 nm using a microplate reader (Bio Rad). The absorption values were calculated using the Microplate Manager 4.0 software (Bio Rad).

2.2.3.10.2 Western blot analysis

The Western blot technique represents a sensitive method to detect specific polypeptides within a complex mixture of proteins. Proteins are separated electrophoretically and transferred to a membrane, which is subsequently incubated with antibodies specific for the protein of interest. Finally, the bound antibody is recognized by a second anti-immunoglobulin that is coupled to horseradish peroxidase or alkaline phosphatase.

Buffer components	**Laemmli buffer (4x)**	**Electrophoresis buffer (1x)**	**Transfer buffer (1x)**	**TBST buffer (10x)**
Tris/HCl	125 mM (pH 6.8)	25 mM	25 mM	100 mM (pH 8.0)
SDS	10% (w/v)	0.1% (w/v)	---	---
Glycine	---	250 mM	192 mM	---
Dithiothreitol	50 mM	---	---	---
Bromophenol blue	0.01% (w/v)	---	---	---
Glycerol	30% (v/v)	---	---	---
Methanol	---	---	20% (v/v)	---
Tween 20	---	---	---	5% (v/v)
NaCl	---	---	---	1.5 M

2.2.3.10.2.1 SDS gel electrophoresis

Electrophoretic separation of proteins was carried out in the discontinuous buffer system for SDS polyacrylamide gels as originally described by Laemmli (1970). 50 µg of total protein were dissolved in 6x Rothi Load (Roth). After heating for 5 min at 95°C, samples were loaded on the gel. Subsequently, the gel was run at a current of 30 mA for a period of 1-3 hours.

Resolving gel

Solution components	Component volumes (ml) per 20 ml gel		
	6% (> 120 kDa)	**10% (60-120 kDa)**	**12% (< 60 kDa)**
H_2O	10.6	7.9	6.6
Acrylamide mix (30%)	4.0	6.7	8.0
Tris/HCl (1.5 M, pH 8.8)	5.0	5.0	5.0
SDS (10%)	0.2	0.2	0.2
Ammonium persulfate (10%)	0.2	0.2	0.2
TEMED	0.016	0.008	0.008

Stacking gel

Solution components	Component volumes (ml) per gel volume of		
	2 ml	**5 ml**	**10 ml**
H_2O	1.4	3.4	6.8
Acrylamide mix (30%)	0.33	0.83	1.7
Tris/HCl (1.5 M, pH 6.8)	0.25	0.63	1.25
SDS (10%)	0.02	0.05	0.1
Ammonium persulfate (10%)	0.02	0.05	0.1
TEMED	0.002	0.005	0.01

2.2.3.10.2.2 Transfer to PVDF membrane

After gel electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting (Trans-Blot SD, Bio Rad). Prior to use, the PVDF membrane was activated in isopropanol for 3 min and subsequently rinsed in deionized water. Six pieces of Whatman 3MM paper were soaked in transfer buffer and positioned on the anode side of the transfer apparatus. The PVDF membrane was placed directly on the stack of 3MM paper. The SDS gel containing the separated proteins was taken off the glasplates, rinsed shortly in transfer buffer, and placed on the top of the PVDF membrane. Finally, the gel was covered with four additional, transfer buffer-soaked Whatman 3MM papers. Air bubbles were squeezed out by a roller apparatus. The upper electrode (cathode) was positioned on the top of the stack and a current of 0,8 mA/cm^2 was applied. Transfer of proteins was carried out at room temperature and terminated after 75 min. After blotting, the membrane was checked by Ponceau S staining for correct electrophoretic transfer and equal loading.

2.2.3.10.3 Staining with Ponceau S

After the protein transfer from gel to membrane always a staining with Ponceau S was done. Purpose of this staining was to determine whether the protein transfer was sufficient and to check for equal loading. Directly after staining the membrane was scanned in its wet condition and thereafter destained by shaking in destilled water.

2.2.3.10.4 Staining with Coomassie-Brilliant-Blue

After protein transfer the gel was stained in Coomassie-Brilliant-Blue (2,5mg/ml Coomassie-Brilliant-Blue G250, 45% Methanol, 45% H_2O und 10% acetic acid). The gel was decoloured after one hour incubation (20% Methanol, 70% H_2O, 10%

acetic acid) for more than 12 hours. This procedure was also done to see if the proteintransfer was sufficient.

2.2.3.10.5 Immunodetection

The air-dried PVDF membrane was reactivated in isopropanol and rinsed in 1x TBST buffer. Non-specific binding sites were blocked by shaking the membrane in a TBST-buffered non-fat skim milk solution (10%) for 1 h at room temperature or overnight at 4°C. The membrane was subsequently exposed to antibodies (diluted 1:500˜ 1:2000 in 1x TBST buffer) specific for the protein of interest. The incubation time varied between 1 h at room temperature, or 15 h at 4°C depending on the antiserum. The blot was washed three times for 15 min in 1x TBST. The primary antibody was detected by incubation of the membrane with a specific secondary antibody coupled to horseradish peroxidase (diluted 1:5000˜ 1:20,000 in 1x TBST) for 45 min at room temperature. For detection of the corresponding bands, we used the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia) according to the instructions of the manufacturer. The membrane was exposed to a film (Hyperfilm, Amersham Pharmacia) and developed (Hyperprocessor, Amersham Pharmacia). Developed films were scanned (GS 700 Imaging Densitometer, Bio Rad) and analyzed using the Molecular Analyst software from Bio Rad.

2.2.3.10.6 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) technique is the most sensitive method to specifically determine protein concentrations from different sources. Sensitivity is approximately 100-fold increased compared to the Western blot technique (*2.7.4*). The ELISA was performed according to the instructions of the manufacturer's (R&D Systems, PharMingen). Briefly, microtiter plates were coated with a monoclonal antibody or a specific receptor. Equal volumes of samples were pipetted into the wells of the microtiter plate and incubated for 2 h. Subsequently, wells were washed. An enzyme-linked antibody specific for the target protein was added for 2 hours, and wells were subsequently washed for three times. Wells were

then incubated with substrate solution. If the samples have been diluted optimal, the color reaction develops in direct proportion to the amount of bound antigen. The optical densities were calculated using the Microplate Manager 4.0 software from Bio Rad.

Used ELISAs:
- Human IL-8 (PharMingen)
- Human IP-10 (PharMingen)
- Human VEGF (R&D)

2.2.3.11 Transfection and Reporter Gene Assays
Plasmid constructs, transient transfection experiments, reporter assays, and mutational analysis

2.2.3.11.1 Reporter Gene Assays

Today gene expression and regulation are often examined by transient- und stable transfection techniques. One method to examine the regulation of the expression of a gene is the reporter gene assay. For this purpose DNA sequenzes in which potential regulatory elements are expected are cloned, in front of a reportergene (in vectors which contain a minimalpromotor). Reporter genes can be the chloramphenicol acetyltransferase (Gorman *et al*.,1982) or the luciferase (Alam and Cook, 1990). The newly cloned reporter constructs are transferred into eukaryontic cells. After transfection the cells are stimulated with the agent of interest. After an appropriate time period that varies depending on the respective gene, cells were harvested and the amount of synthesized reporter gene products was measured. To clarify the influence of the investigated sequence on the genregulation it is necessary to compare the expression of the reporter gene with and without the putative regulatory sequence in the plasmid.

2.2.3.11.2 Transfection of eukaryontic cells

Several methods are used to transfere DNA into eukaryontic cells. Mostly applied methods are the microinjection, the calcium phosphate transfection, the DEAE-

Dextran transfection, the electroporation, and the transfection by help of liposomes (artificial membrane vesikel). Substances which could enclose DNA like Lipofectamin (Gibco, Eggenstein) or FuGENE 6 (Roche Boehringer) consist of artifical, neutral and kationic lipids. The efficacy with which the liposome-nucleic acid-complexes were taken up has a strong variation rate dependending on the cell line in use. For this purpose for each cell line a suitible reagent with an individual protocol must be established. The molecular mechanisms of transfection are largely unknown. Probably the liposomal complex is taken up by endocytosis into the cell followed by the transport of DNA from the cytoplasma in to the nucleus.

2.2.3.11.3 Transient transfection

Confluent grown cells were trypsinized and cultured in fresh media. The cells should have a confluency of about 50-60%. The cells were seeded in 2ml media per well onto six-well-culture-plates and incubated for 18h in an incubator with standard conditions. Luciferase expression contructs (750 ng DNA/35 mm-well ultra pure) were transfected into DLD-1 cells using FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's instructions we used 3µl of Fugene Transfection Reagent per well. Plasmid DNA and 3µl FuGENE 6 Reagent were diluted with 300 µl serum-free medium, combined and mixed gently. The solutions were allowed to form DNA-liposome complexes for 30 min at room temperature. Target cells were rinsed two times with PBS and added fresh media. The DNA-liposome complexes were added and incubated with the 2 ml of the diluted DNA-liposome complex for 36h without replacing the transfection mixture. For control of transfection efficiency 500 ng of pCMV-β galactosidase DNA was cotransfected. After a 36 h transfection period, cells were washed twice with PBS. DLD-1 cells were then either kept as unstimulated control or stimulated with PDTC or TNFα. After 8h, cells were harvested and luciferase activity was determined using the Promega Luciferase Assay System (Promega, Madison).

2.2.3.11.4 Construction of the IL-8 promotor and its mutants

The human IL-8 promotor 5'-flanking region from -558 to +98 bp was amplified by *pfu*-polymerase (Stratagene, Gebouw California, Netherlands) from human genomic DNA using the oligonucleotides 5'-ctt cac tct gtt aac tag cat ta-3' (-558/-535) as forward and 5'-aca cac agt gag aat ggt tcc t-3' (+76/+98 bp) as reverse primer, respectively. The resulting PCR product was cloned into pGL3-promotor vector containing a *luc*+transcriptional unit. Constructs were verified by sequencing. Mutations were performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. For the mutations of the AP-1, NF-κB, and C/EBPβ sites (Vlahopoulos, S., et al. 1999) the following primer sequences were used: activator protein-1 (AP-1) sequence (forward) 5'-aag tgt gat ***at***c tca ggt ttg ccc tga-3', (revers) 5'-caa acc tga g***at*** atc aca ctt cct a-3'; NF-κB sequence (forward) 5'-ttg caa atc gt***t tt***a att t***aa*** tct gac ata a-3', (revers) 5'-ttc att atg tca ga***t t***aa att ***aaa*** cga ttt-3'; C/EBPβ sequence (forward) 5'-gcc atc ag***c*** t***a***c ***g***a***g*** tcg tg-3', (reverse) 5'-gaa att cca cga ***c***t***c*** g***t***a ***g***ct-3'. Mutations were checked by sequencing using the automated sequence analyzer ABI 310 Genetic Analyzer (PE Applied Biosystems, Weiterstadt, Germany).

2.2.3.11.5 Determination of reporter gene induction

The luciferase of the Firefly (*Photinus pyralis*) catalyzes the oxidation of luciferin. During this reaction photons at a wavelength of 562 nm were emitted. A quantitative measurement of the emitted light is possible by use of a Luminometer. Cells were transiently transfected with a luciferase-construct, stimulated, and harvested in Reporter-Lysisbuffer according to the manual of Promega. The solutions which were needed were prewarmed to room temperature. 20 µl of cell extrakt was pipetted in a polystyrole tube. These were transfered into the Luminometer. The measurement was performed, after injection (in the Luminometer) of 100 µl Luciferase Assay Reagent (Promega) (20 mM Tricin, 1,07 mM $(MgCO_3)Mg(OH)_2$ x 5 H_2O, 2,67 mM $MgSO_4$, 0,1 mM EDTA, 33,3 mM DTT, 270 µM Coenzym A, 470 µM Luciferin, 530 µM ATP pH 7,8). The emitted light was

measured for 10 sec with a Photomultiplier. Data were expressed as relative Light Units (RLU).

2.2.3.11.6 Detection of ß-Galactosidase expression

The ß-galactosidase (β-gal) is a bacterial enzyme which catalyzes the break down of the sugar lactate. This enzyme is encoded by the bacterial Gen *lacZ* located in the lac-Operon of ß-gal. Lactate is not the only substrate wich can be catalysed. An alternative substrate that is often used is 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Its enzymatic degradation by β-gal leads to blue colored products. The resulting blue stain of the reaction can be quantified. The *lacZ* Gen under the control of a constitutive promotor was transfected into DLD-1 cells to determine the transfection efficacy. Analysis was performed by two different staining methods as outlined below.

2.2.3.11.7 Histochemical Staining of ß-Gal positive cells

This method was used to establish the transfection method. The advantage of this method is that each single cell which is positively transfected could be detected by microscopy. The cytosol of positive transfected cells gets blue after histochemical treatment. So that the efficacy ot transfection could simply be detected by counting the blue cells.
Protocol of treatment:
The cells were first washed with PBS and then fixed with glutaraldehyde. For a 6cm culture dish 3ml fixing and staining solution were used.

The fixing solution (10ml) :
9,6 ml PBS
284 µl Formaldehyde 37% (1% final concentration)
100 µl Glutaraldehyde 25% (0,25% final concentration)

After 5min incubation the fixing solution was totally removed and cells were washed two times with PBS. Afterwards the staining solution was added. Fixing and staining solutions have to be prepared freshly before the experiment.

Staining solution (10ml):
8.75 ml PBS
1ml Ferro-Ferri (final concentration 10%)
250µl X-Gal-solution (final concentration 2.5%)
25µl $MgCl_2$ (1M, final concentration 0.25%)

The cells were transfered into the incubator with the staining solution for several hours. After a few hours in positive cells a blue color becomes visible.
For longer storage the staining solution was removed and replaced by 2% Glycerol. The dishes were then enclosed with parafilm and stored in the refrigerator.

2.2.3.11.6 Proof of ß-Gal in lysates of transfected cells

This method is used to measure the amount of ß-gal in lysates of transfected cells of luciferase assays. The transfection efficacy varies in one experiment upon the transfected cells in different wells of the dishes. For control of transfection efficiency a constant amount of pCMV-β galactosidase DNA was cotransfected with firefly constructs of interest. The pCMV-β galactosidase plasmid contains a constitutive promotor which delivers a constant expression of β-gal. The determined amount of β-gal was used to set off against the RLUs' out of the luciferase assays (see 2.2.3.11.5).

Buffers and solutions:

100 x Mg^{2+}-buffer	100 mM $MgCl_2$
	5 mM 2- mercaptoethanol

0.1 M Sodium phosphate-buffer	100 ml of 0.1 M Na_2HPO_4 adiusted to pH 7.3 at 37^{O}C using 0.1 M NaH_2P0_4
ONPG-solution	4 mg/ml in O,1 M Sodium phosphate-buffer
Stop-solution	1 M Na_2CO_3 solution

The following reagents were combined:
10-150 µl of cell extract (extraction for ß-Galactosidase was performed as previously described in 2.2.3.11.5 with promega lysis buffer), 3 µl of
100 x Mg-buffer, 66µl ONPG solution and Sodium phosphate-buffer were added to a final volume of 300 µl. The reaction- was incubated at 37°C for 30 min to several hours until a yellow color was visible. The reaction was stopped by adding 0.5 ml of stop solution. The color reaction was measured photometrically at 410 nm.

2.2.3.12 Electrophoretic mobility shift assay (EMSA)

2.2.3.12 Detection of proteins using EMSA analysis

The EMSA method is to characterize the interaction between a protein and a DNA double strand. The gel-shift method relies on the binding of an specific ^{32}P labeled oligonucleotide to a particular DNA binding protein. The nucleotide/protein complexes can be separated from the free oligonucleotide on a non denaturating acrylamide gel. In contrast to the Western blot method a denaturating gel can not be used, because for the interaction of the DNA with the protein, native structure is necessary. Proof of the identity of the DNA binding protein can be achieved by supershift analysis. In this approach an antibody which is known to bind specifically to a protein of interest is added to the reaction mix. If the antibody successfully bind to the DNA protein complex the so called supershift may become visible on the gel which is due to the higher molecular weight of the antibody/antigen/DNA complex. Alternatively, the respective band dissappears if the antibody interferes with the DNA binding site of the protein of interest.

2.2.3.12.2 Nuclear extrakts

Subconfluent cells on 10 cm dishes were stimulated for 30 min to 4h washed twice with cold PBS and scraped in ice cold 1.5 ml PBS/EDTA (0,1mM (pH8,0)). All steps were performed strictly in a cold enviroment (lower than 4°C). The cells were pelleted by centrifugation in an cold centrifuge at 13000 rpm for 5 min. The pellet was resuspended in 300 µl cold buffer A + 0.6%NP40 + protease inhibitor and incubated for 15 min by vigorous shaking at 4°C. Nuclei and cell fragments were pelleted by centrifugation in a centrifuge at 13000 rpm and 4°C for 15 min. The supernatant consists of a cytosolic extract which could be used for Western blot analysis. The DNA binding proteins in their active form are located in the nucleus. Thus the nuclear pellet has to be resuspended in 70µl ice cold buffer C + protease inhibitor and incubated for 20 min by vigorous shaking at 4°C. Nuclei and cell fragments were pelleted by centrifugation in a centrifuge at 13000 rpm and 4°C for 5 min. Finally, the supernatant contains the nuclear extracts. Protein concentrations were measured as described bevore by the Bradford method. The protein solutions were aliquoted, shock frozen in liquid nitrogen and stored at –80°C until use.

2.2.3.12.3 Labeling of oligonucleotides

The consensus oligonucleotides which were used in the binding reactions were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Sequences of the double-stranded oligonucleotides are as follows, NF-κB: wt 5'-agt tga ggg gac ttt ccc agg c-3'; mutated 5'-agt tga gg***c*** gac ttt ccc agg c-3'. AP-1: wt 5'-cgc ttg atg act cag ccg gaa-3'. Complementary oligonucleotides were end labeled by T4 polynucleotide kinase (MBI Fermentas, St. Leon-Roth, Germany) using [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech, Braunschweig, Germany).

Oligolabeling:	2 µl	ds Oligo (pgml/µl) or 2µl consensus
	2 µl	10x Polynucleotide Kinase Buffer
	10 µl	H_2O

1 µl T4 Polynucleotidekinase

5 µl [γ-^{32}P]ATP

The reaction mix was incubated for 1h at 37°C. Thereafter the enzyme was heat inactivated at 75°C for 10 min and slowly cooled down overnight . The labeled probe was cleaned up by using Nick Colums (Amersham, Pharmacia) due to the manufactors' protocoll. The incorporated activity of the freshly prepared radiolabeled probe was determined using a β-counting device (TRI-CARB 2100 TR, Canberra-Packard).

2.2.3.12.4 Binding reaction, analytical gel electrophoresis and signal detection

Binding reactions were performed for 35 min on ice with 10 μg of protein in 20 μl of binding buffer containing 4% Ficoll, 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.25 mg/ml BSA, 2 μg of poly(dI-dC), and 20,000-25,000 dpm of ^{32}P-labeled oligonucleotide. For AP-1 super - shift analysis nuclear proteins were preincubated for 15 minutes at room temperature with a polyclonal anti-c-jun antibody (Santa Cruz, CA). DNA protein complexes were separated from unbound oligonucleotide by electrophoresis through a 4% polyacrylamide gels using 0.5 x TBE buffer.

EMSA Gel

Solution components	**Component volumes (ml) per 36 ml gel**		
	4,5%	**6%**	**8%**
H_2O	28,3	27	24,1
Acrylamide mix (30%/0,8%)	5,4	7,2	9,6

10 x TBE	1,8	1,8	1,8
Ammonium persulfate (10%)	0,4	0,4	0,4
TEMED	0,3	0,3	0,3

Thereafter, gels were fixed and analyzed by PhosphoImager analysis (Fuji). Competition experiments were performed by coincubation with a 100-fold excess (20 pmol) of unlabeled double-stranded oligonucleotide in the DNA-protein binding reaction.

3 Results

3.1.1 PDTC efficiently augments TNFα-induced IL-8 release and mediates production of IL-8 as a single stimulus in DLD-1 cells

While studying the expression of IL-8 in DLD-1 colon carcinoma cells, we investigated the effects of PDTC, a compound which is widely used as inhibitor of NF-κB activation. Unexpectedly, PDTC not only efficiently augmented TNFα-induced IL-8 (Fig.6A), but was also able to mediate release of IL-8 from DLD-1 cells as a single stimulus (Fig. 6A&B). Under these experimental conditions induction of IL-8 was not associated with cytotoxicity as assessed by quantitative analysis of DNA-fragmentation, a common marker of apoptotic cell death. Treatment with the antioxidant acetylcystein did not mediate IL-8 expression (data not shown). Time-course analysis revealed that PDTC induced a continous release of IL-8 from the cells that started after 4h of incubation. Most of the IL-8 was released between hour 8 and 24 of stimulation. After 24h, a plateau of IL-8 concentration in the culture supernatants was reached (Fig.6C).

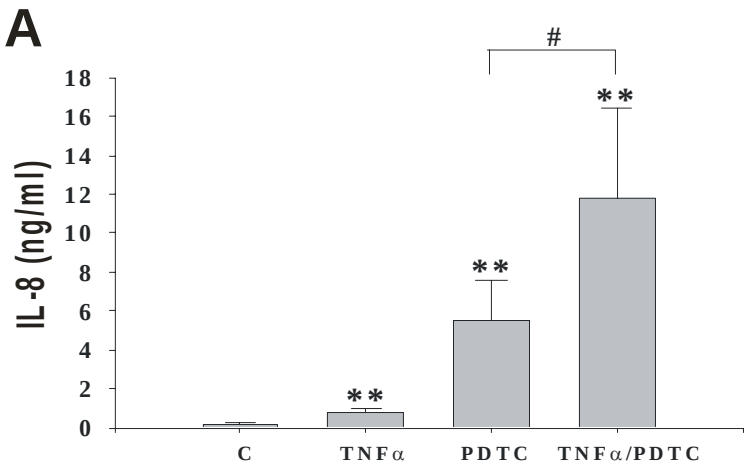


Fig. 6: (A) PDTC enhances TNFα-induced release of IL-8 from DLD-1 cells and likewise induces IL-8 release as single stimulus. DLD-1 cells were incubated as unstimulated control (C), stimulated with TNFα (50 ng/ml), with PDTC (50 μM), or with TNFα (50 ng/ml) in combination with PDTC (50 μM) as indicated. After 24h, cell-free supernatants were assayed for IL-8 protein content by ELISA. Data are expressed as mean IL-8 concentrations ± SD (n = 4). **, $p < 0.01$ compared to untreated control; #, $p < 0.05$ compared to PDTC alone.

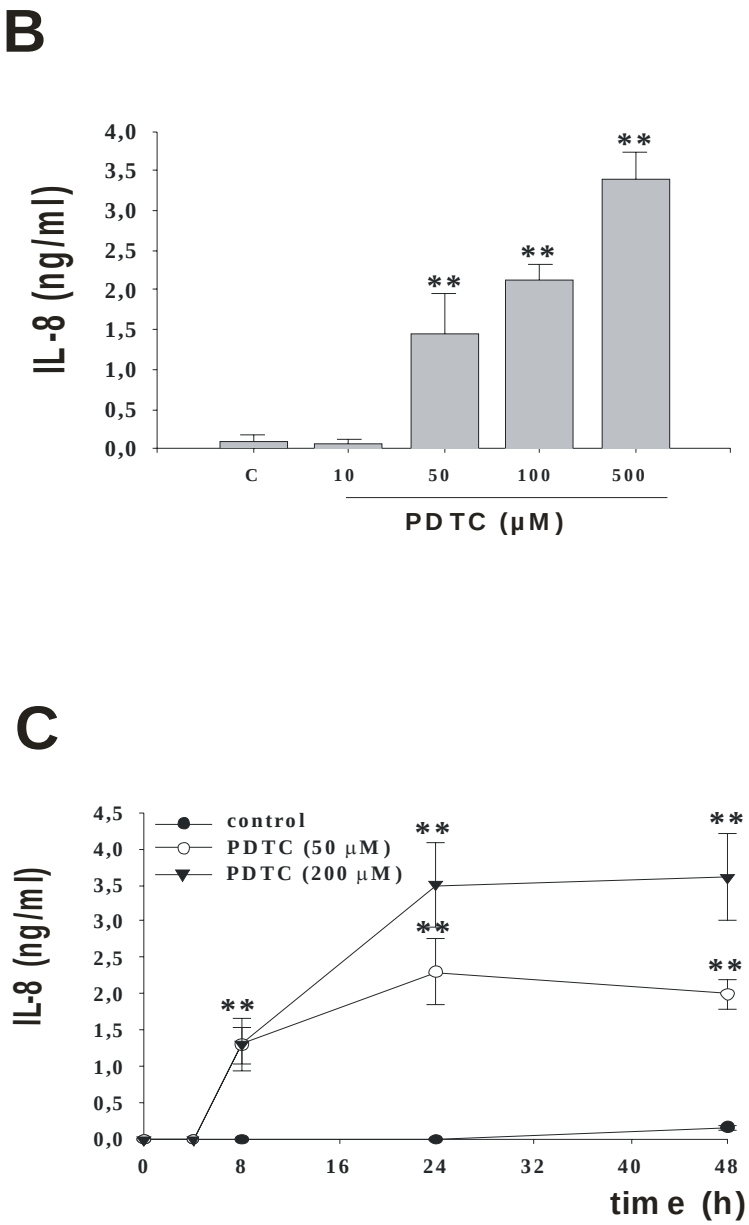


Fig. 6: (B,C) PDTC enhances TNFα-induced release of IL-8 from DLD-1 cells and likewise induces IL-8 release as single stimulus. (B) DLD-1 cells were incubated as unstimulated control (C) or stimulated with the indicated concentrations of PDTC. After 8h, cell-free supernatants were assayed for IL-8 protein content by ELISA. Data are expressed as mean IL-8 concentrations ± SD (n = 5). **, $p < 0.01$ compared to untreated control. (C) DLD-1 cells were incubated as unstimulated control (closed circles) or stimulated with PDTC at 50 µM (open circles) or 200 µM (closed triangles). After the indicated time periods, cell-free supernatants were assayed for IL-8 protein content by ELISA. Data are expressed as mean IL-8 concentrations ± SD (n = 4). **, $p < 0.01$ compared to untreated control.

3.1.2 PDTC induces gene expression of IL-8, VEGF, and HO-1 in DLD-1 cells

To further characterize PDTC-induced activation of DLD-1 cells, mRNA accumulation in response to the agent was investigated by RNase protection assay. In addition to IL-8, expression levels of two additional markers of cellular activation were determined: VEGF and HO-1 mRNA induction by PDTC was observed for all three genes investigated (Fig.7A-C). In order to confirm that upregulation of mRNA levels translated into protein expression, immunoblotting for HO-1 was performed. As shown in Fig.8, PDTC was a strong inducer of HO-1 in DLD-1 cells. As a positive control, DLD-1 cells were exposed to the nitric oxide donor GSNO, which was previously identified as mediator of HO-1 expression in rat renal mesangial cells (Sandau, K., et al. 1998) and human HaCaT keratinocytes (Wetzler C., et al. 2000). In contrast, stimulation of DLD-1 cells with the proinflammatory cytokine IL-1β was not sufficient to trigger induction of HO-1, which is in accord with a recent publication on regulation of HO-1 in HaCaT keratinocytes (Hanselmann, C., et al. 2001).

A

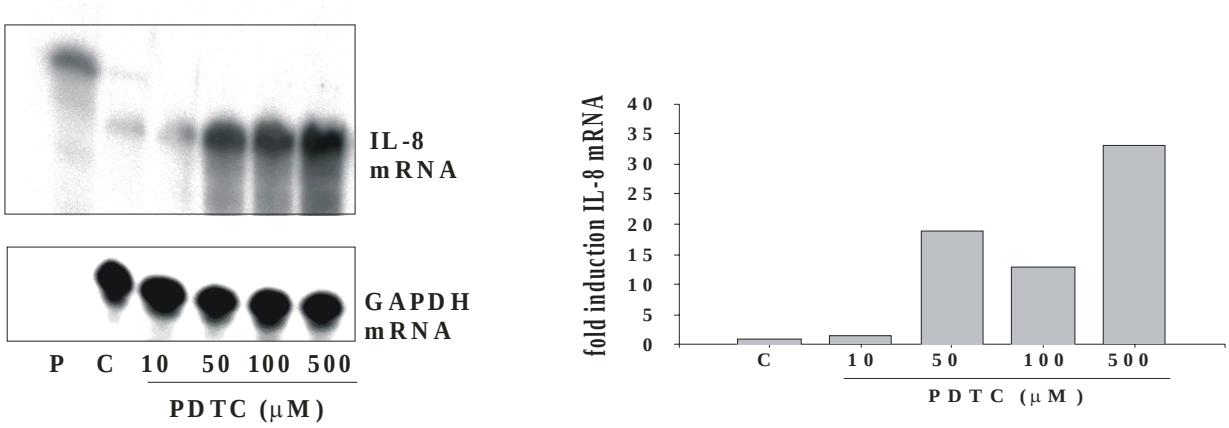
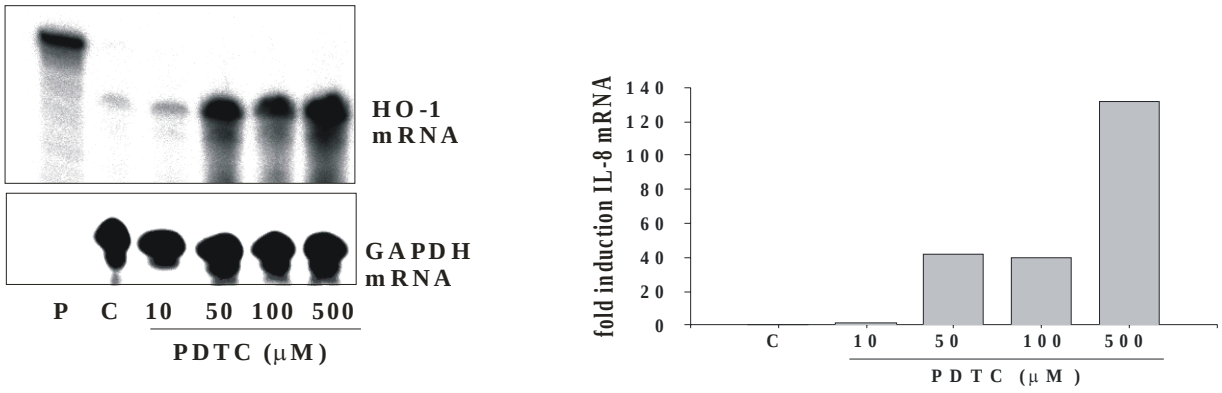


Fig. 7: (A) PDTC induces gene expression of IL-8, HO-1, and VEGF in DLD-1 cells. DLD-1 cells were incubated as unstimulated control (C) or stimulated with the indicated concentrations of PDTC for 8h. Thereafter, PDTC-induced IL-8 (A) mRNA accumulation was evaluated by RNase protection assay. One representative of three independently performed experiments is shown. Relative mRNA expression of this same experiment was quantified by PhosphoImager (Fuji) analysis of the radiolabeled gel. P denotes probe.

B



C

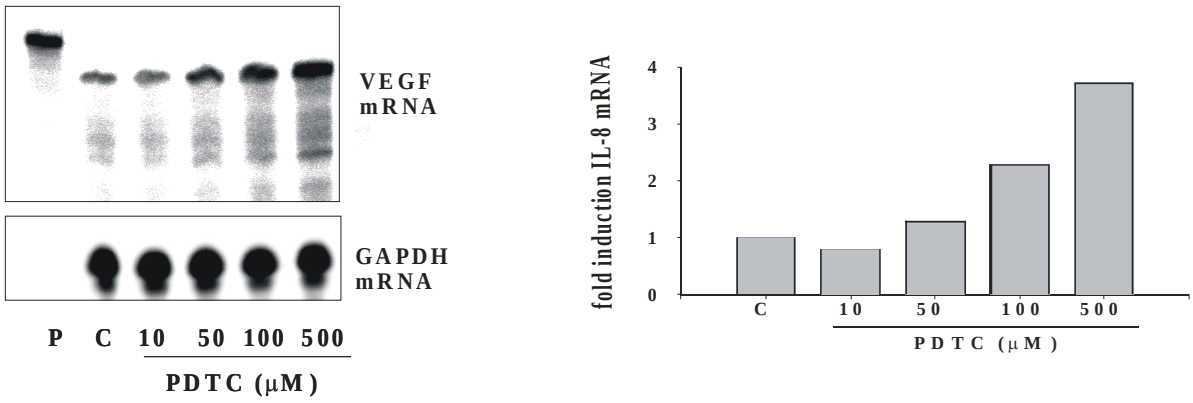


Fig. 7: (B,C) PDTC induces gene expression of IL-8, HO-1, and VEGF in DLD-1 cells. DLD-1 cells were incubated as unstimulated control or stimulated with the indicated concentrations of PDTC for 8h. Thereafter, PDTC-induced HO-1 (B), and VEGF (C) mRNA accumulation was evaluated by RNase protection assay. One representative of three independently performed experiments is shown. Relative mRNA expression of this same experiment was quantified by PhosphoImager (Fuji) analysis of the radiolabeled gel. P denotes probe.

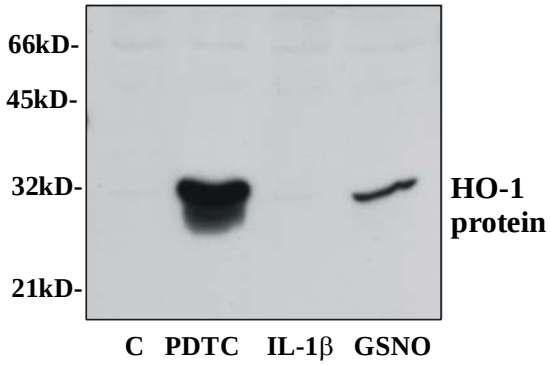
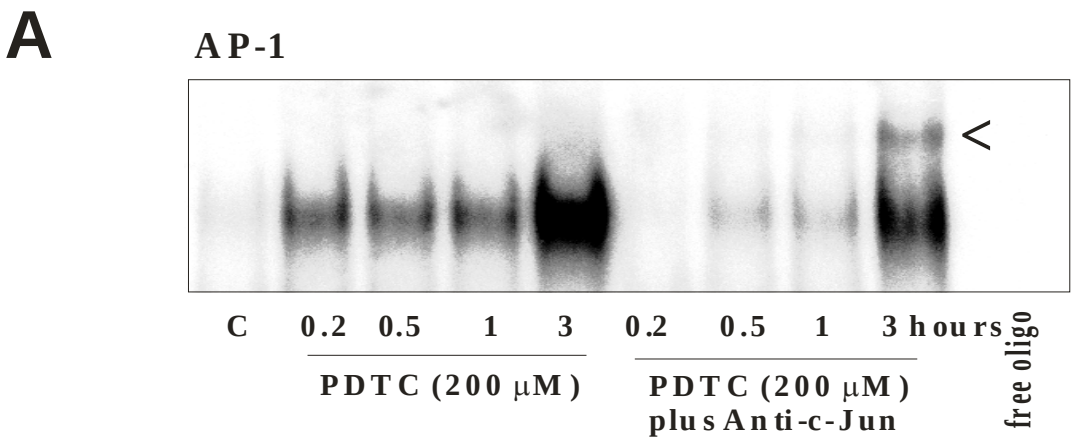


Fig. 8: PDTC induces expression of HO-1 protein in DLD-1 cells. DLD-1 cells were incubated as unstimulated control or stimulated with PDTC (50 μM), IL-1β (50 ng/ml), or GSNO (500 μM) as indicated. After 8h, cells were harvested and homogenates were assayed for the presence of HO-1 protein by immunoblotting.

3.1.3 PDTC upregulates IL-8 promotor activity in DLD-1 cells through an NF-κB- and AP-1-dependent mechanism

Gene induction of proteins such as intercellular adhesion molecule-1 (Munoz, C., et al. 1996), β2-integrin (Aragones, J., et al. 1996), stromelysin (Yokoo, T. and Kitamura, M. et al. 1996), or glutathione S-transferase (Pinkus, R., et al. 1996) by PDTC is mediated by activation of the transcription factor AP-1. In keeping with these previous observations, we observed activation of AP-1 by PDTC by performing EMSA analysis (Fig.9A). In order to further investigate whether activation of the IL-8 promotor was responsible for induction by PDTC, we transiently transfected the luciferase reporter gene under the control of a IL-8 promotor fragment (-558 to +98 nt) (10) into DLD-1 cells. To evaluate a potential role for the AP-1 binding site (-127 to –120 nt) in the IL-8 promotor, a site-directed mutation of this site was performed. In addition, a NF-κB mutant (-80 to –70) was generated in the same context of the IL-8 promotor fragment (-558 to +98 nt). As a positive control for activation of the IL-8 promotor, DLD-1 cells were stimulated with TNFα. As expected, TNFα induction of IL-8 promotor activity was abbrogated by mutation of the NF-κB binding site in the IL-8 promotor fragment (Fig.9C). In agreement with PDTC-induced IL-8 mRNA accumulation and protein release, promotor activity was significantly upregulated by the agent (Fig.9C). Mutational analysis revealed that both, AP-1 and NF-κB were necassary for induction of IL-8 by PDTC (Fig.9C). It is well described that AP-1 and NF-κB can synergistically activate the IL-8 promotor (Roebuck, K.A. 1999). Therefore, we investigated the status of NF-κB activation in DLD-1 cells. A specific DNA-binding complex was strongly upregulated in response to TNFα, which was

used as a positive control in these experiments. In accord with a previous report (Kleinert, H., et al. 1998)), constitutive NF-κB binding activity was observed in DLD-1 cells. This constitutive NF-κB binding activity was still detectable in the presence of PDTC (Fig. 9B). It has been reported previously that PDTC is able to activate the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) (Chinery, R., et al. 1997)). In addition, C/EBPβ has been identified as costimulus for optimal IL-8 induction (Roebuck, K.A. 1999). To evaluate a potential role for the C/EBPβ binding site (-94 to -81 nt) in PDTC-induced IL-8 promotor activation, a site-directed mutation was performed, in the same context of the IL-8 promotor fragment (-558 to +98 nt). As shown in Fig. 9C, mutation of this site only partially reduced PDTC-induced IL-8 promotor activity.



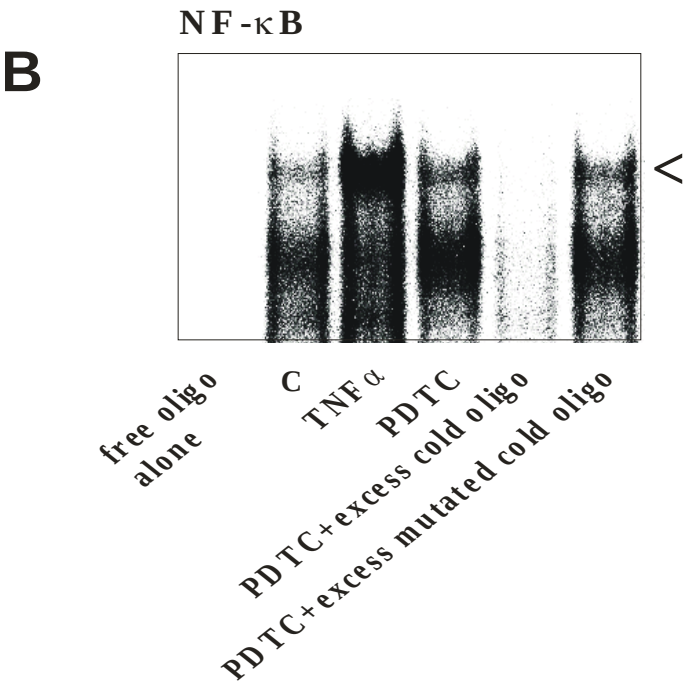


Fig. 9: (A,B) PDTC upregulates IL-8 promotor activity in DLD-1 cells through an NF-κB- and AP-1-dependent mechanism. (A) Detection of PDTC-induced AP-1 DNA binding activity in DLD-1 cells by EMSA analysis. DLD-1 cells were incubated as unstimulated control (C), or stimulated with PDTC (200 μM) for the indicated time periods. Thereafter, nuclear extracts were prepared and EMSA analysis was performed using a ^{32}P-labeled AP-1 consensus oligonucleotide.
Experiments were performed in the presence or absence of an anti-c-Jun antibody. The position of a supershifted band is indicated by an arrow. One

representative of three independently performed experiments is shown. (B) Detection of constitutive DNA binding activity of NF-κB in DLD-1 cells by EMSA. DLD-1 cells were incubated as unstimulated control (C), or stimulated with TNFα (50 ng/ml), or PDTC (50 μM) as indicated. After 1h, nuclear extracts were prepared and EMSA analysis was performed using a ^{32}P-labeled NF-κB consensus oligonucleotide, excess cold NF-κB consensus oligonucleotide, and excess cold mutated NF-κB consensus oligonucleotide as indicated. One representative of two independently performed experiments is shown.

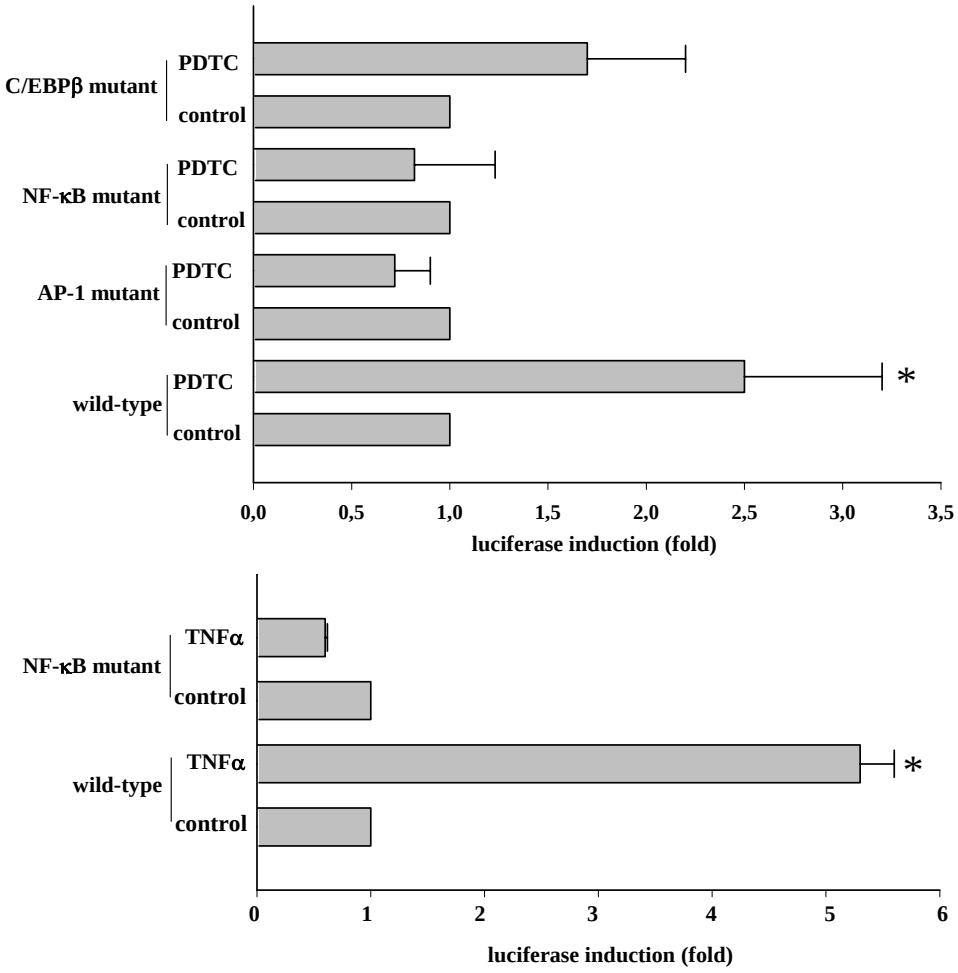


Fig. 9: (C) PDTC upregulates IL-8 promotor activity in DLD-1 cells through an NF-κB- and AP-1-dependent mechanism. 500 ng/well of β-Gal-constructs and 500 ng/well of one of the indicated luciferase-IL-8-promotor-constructs were used to transiently transfect DLD-1 cells. After 36h, transfected cells were either maintained as unstimulated control, or treated with PDTC (50 μM) (upper panel), or with TNFα (50 ng/ml) (lower panel) for additional 8h. Thereafter, cells were harvested and luciferase and β-Gal assays were performed, respectively. Data are expressed as fold induction of luciferase and are means ± SD (n = 5). *p < 0.05 compared to unstimulated control. A one-fold

induction was assigned to the luciferase activity versus β-Gal activity in the respective unstimulated transfected DLD-1 cells.

3.1.4 PDTC augments release of IL-8 from PBMC

We also studied the effect of PDTC on IL-8 release from PBMC. As shown in Fig.10A, basal release of IL-8 from PBMC was significantly augmented by incubation with PDTC. In order to control for a potential LPS-contamination of the agent, experiments using the inhibitor of LPS action polymyxin B (PmxB) were performed. Upregulation of IL-8 release was not affected by pretreatment of control medium and PDTC with PmxB (final concentration: 2μg/ml) (Fig. 10A). Under the same experimental conditions LPS (10 ng/ml)-induced release of IL-8 from PBMC was efficiently abbrogated by pretreatment with PmxB (Fig.10B). LPS at 100 ng/ml did not mediate release of IL-8 from DLD-1 cells (data not shown).

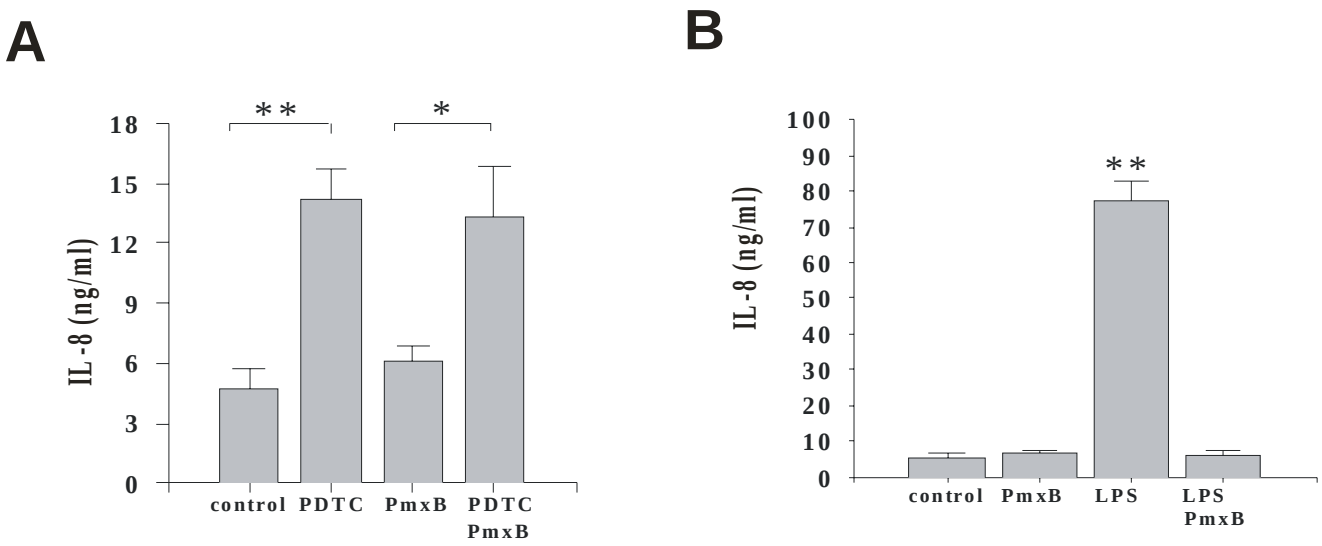


Fig. 10: PDTC augments release of IL-8 from PBMC. PBMC were incubated as unstimulated control (A,B), or stimulated with PDTC (50 μM) (A), or with LPS (10 ng/ml) (B). Cells were also incubated as PmxB pretreated (final concentration: 2 μg/ml) control (A,B), or stimulated with PmxB pretreated (final concentration: 2 μg/ml) PDTC (50 μM) (A), or with PmxB pretreated (final concentration: 2 μg/ml) LPS (10 ng/ml) (B). After 24h, cell-free supernatants were determined by ELISA. Data are expressed as means ± SEM (n = 4). *p < 0.05 compared to PmxB pretreated control; **p < 0.01 compared to untreated or PmxB pretreated control.

3.1.5 PDTC augments release and induces gene expression of IL-8 in U937

We also studied the effect of PDTC on IL-8 release in U937 cells. As shown in Fig.11A-C, basal release of IL-8 from U937 was significantly augmented by incubation with PDTC. To further characterize PDTC-induced activation of U937 cells, mRNA accumulation and IL-8 release in response to the agent was investigated by RT-PCR and ELISA, respectively. As shown in Fig. 11, expression and release of IL-8 was observed in U937 cells exposed to PDTC.

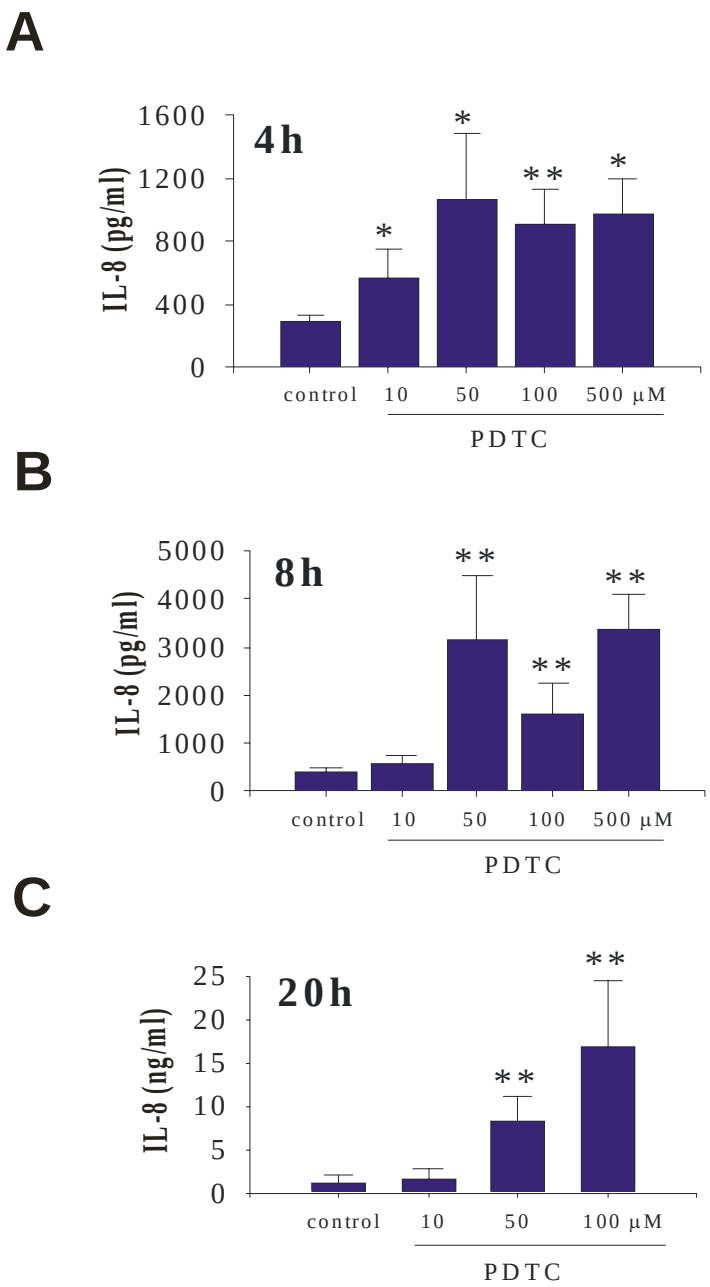


Fig. 11: (A-C) PDTC induces IL-8 release from U937 cells. (A-C) U937 cells were incubated as unstimulated control, or stimulated with PDTC (10-500 µM). After 4h,8h, and 20h, cell-free supernatants were assayed for IL-8 protein content by ELISA. Data are expressed as mean IL-8 concentrations ± SD (n = 4). **p < 0.01 compared to untreated control.

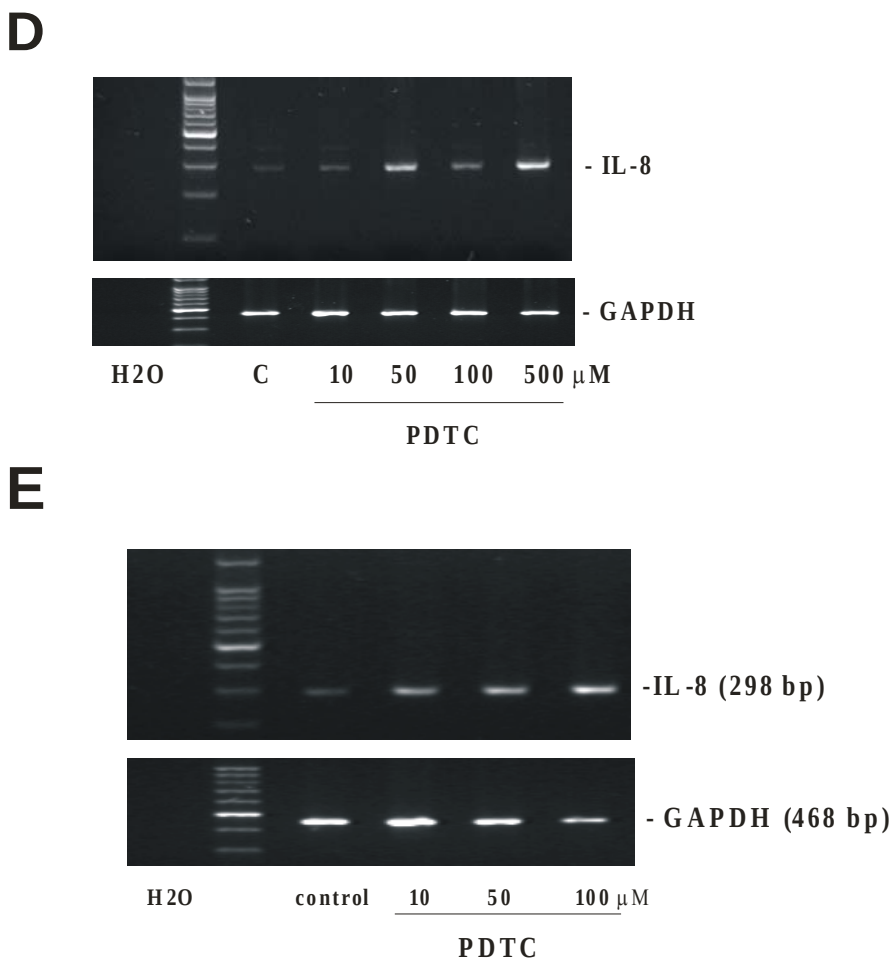


Fig. 11: (D,E) PDTC induces gene expression of IL-8 in U937 cells. (D, E) U937 cells were incubated as unstimulated control or stimulated with the indicated concentrations of PDTC for 8h (D) or 20h (E). Thereafter, PDTC-induced IL-8 mRNA accumulation was evaluated by RT-PCR. One representative of three independently performed experiments is shown.

3.2.1 Induction of iNOS in DLD-1 colon carcinoma cells

Induction of iNOS protein and activity has been observed in colorectal cancer (Ambs, S., 1998 and 1999; Szaleczky, E., et al. 2000) and is supposed to be mediated by inflammatory cytokines which are expressed at the tumor site, among others IL-Iβ, TNFα, and IFNγ (Csiszar A., et al. 2001; Numata A, et al. 1991; Yoshimi N., et al. 1994) . Accordingly, activation of DLD-1 colon carcinoma cells by exogenous IL- 1 β/IFNγ resulted in strong expression of iNOS (Fig. 12).

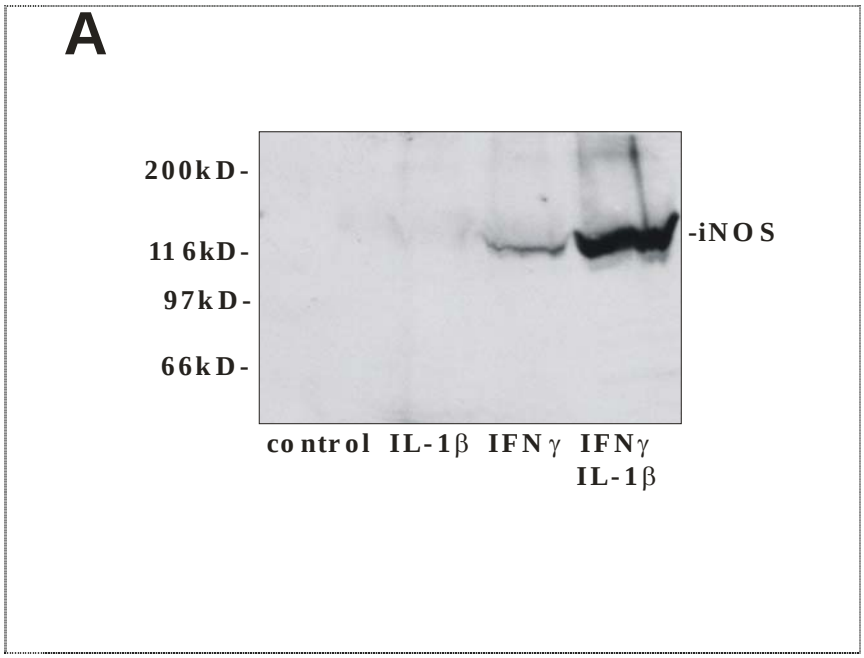
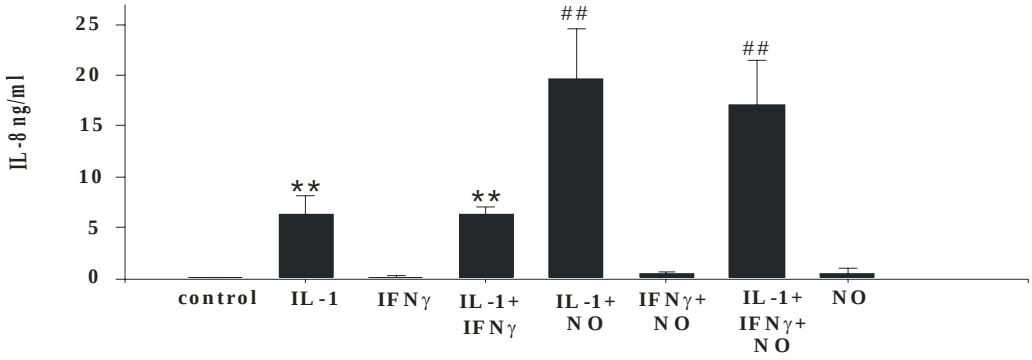


Fig. 12 Confluent DLD-1 cells were stimulated for 48h with IL-1β (50 ng/ml), IFN-γ (20 ng/ml), with IL-1β (50 ng/ml) plus IFNγ (20 ng/ml), or incubated as unstimulated control. Thereafter, iNOS protein expression was investigated by immunoblot analysis. One representative of two independently performed experiments is shown.

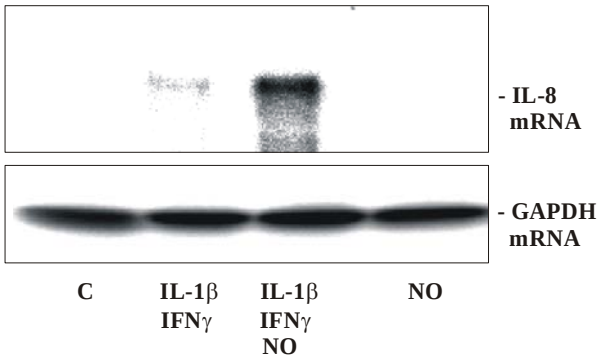
3.2.2 Nitric oxide amplifies expression of proangiogenic IL-8 and VEGF in DLD-1 colon carcinoma cells.

IL-8 has been recognized as an important proangiogenic mediator (Belperio, J.A., et al. 2000) and a proliferative stimulus for colon carcinoma cells (Miyamoto, M. et al. 1998). Thus, effects of exogenous NO on the production of IL-8 by DLD-1 cells were investigated. As shown in Fig.13, IL-1β potently activated release of IL-8. Coincubation with IL-1β/IFNγ did not further enhance IL-8 production. Moreover, IL-8 was not inducible by IFNγ alone. Thus, IL-8 is primarily an IL1 β-dependent gene in this experimental system. The role of NO in regulating gene expression in these cells was investigated by preincubation of cells with the NO-donor DETA-NO which spontaneously releases NO with a characteristically long half-life of 16.5h (Mühl H. et al., 2000). DETA-NO significantly augmented release of IL- 8 by DLD- 1 cells stimulated either with IL- 1 β alone or with the combination IL- 1 β/IFNγ. Augmented secretion of IL-8 protein under the influence of NO was paralleled by upregulation of IL-8 mRNA steady-state levels (Fig.13B). NO also significantly increased low level basal release of IL-8 protein (Fig.13C) and expression of mRNA as detected by RT-PCR analysis (data not shown). In accord with previous data we confirm that DLD-1 cells constitutively produce VEGF (Okada, F., et al. 1998). Basal expression of VEGF was significantly upregulated by coincubation with NO (Fig. 14).

A


IL-8 ng/ml
25
20
15
10
5
0
**
**
##
##
control
IL-1
IFNγ
IL-1+ IFNγ
IL-1+ NO
IFNγ+ NO
IL-1+ IFNγ+ NO
NO

B


- IL-8 mRNA
- GAPDH mRNA
C
IL-1β IFNγ
IL-1β IFNγ NO
NO

C

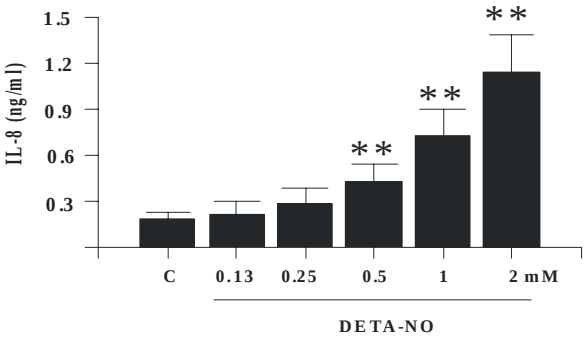

IL-8 (ng/ml)
1.5
1.2
0.9
0.6
0.3
**
**
**
C
0.13
0.25
0.5
1
2 mM
DETA-NO

Fig. 13: (A-C) NO enhances expression and release of IL-8 from DLD-1 colon carcinoma cells. (A) DLD-1 cells were incubated as unstimulated control or with DETA-NO (NO, 1 mM). After 16h, IL- 1β (50 ng/ml), IFNγ (10 ng/ml) or IL- 1β plus IFNγ were added as indicated. Following an additional 8h incubation period, release of IL-8 was determined by ELISA. Mean IL-8 concentrations ± SD are shown (n = 6). **, $p < 0.01$ compared to unstimulated control; ##, $p < 0.01$ compared to stimulation with IL-1β/IFNγ alone. (B) DLD-1 cells were incubated with control culture medium (C) or with DETA-NO (NO, 1 mM). After 16h, IL-1ß (50 ng/ml)/IFNγ (10 ng/ml) were added. Following an additional 8h incubation period, expression of IL-8 was determined by RNase protection assay. One representative of three independently performed experiments is shown. (C) NO as a single stimulus mediates expression of IL-8. DLD-1 cells were stimulated with the indicated concentrations of DETA-NO. After 25h, expression of IL-8 was determined by ELISA (C). Data are expressed as mean IL-8 concentrations ± SD (n6). **, $p < 0.01$ compared to unstimulated control.

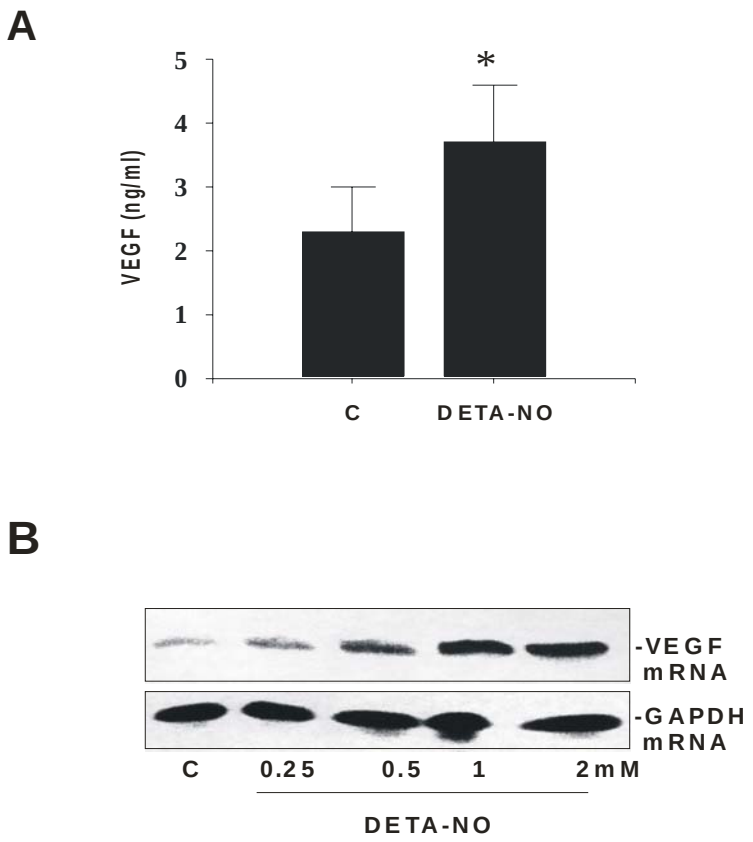
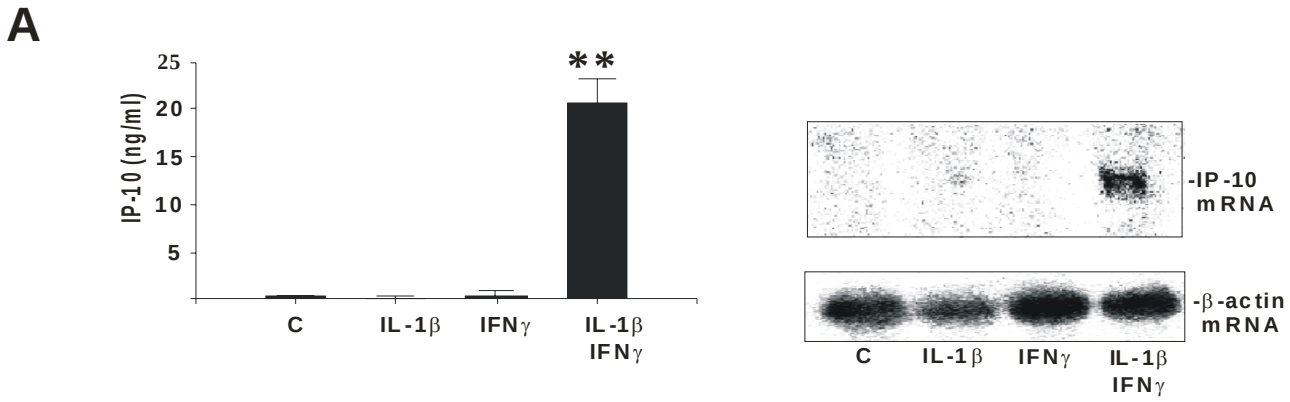


Fig. 14: (A,B) NO mediates induction of VEGF in DLD-1 cells. DLD-1 cells were stimulated for 24h with DETA-NO at 1 mM (A) or with DETA-NO in the indicated concentrations (B). Thereafter, release of VEGF was determined by ELISA (A). Data are expressed as mean VEGF concentrations ± SD (n = 6). *, $p < 0.05$ compared to unstimulated control. mRNA expression of VEGF was evaluated by RNase protection assay (B).

3.2.3 Nitric oxide suppresses IL-1β/IFNγ-induced expression of the antiangiogenic chemokines IP-10 and MIG as well as expression of iNOS

Among the chemokines expressed by IL-1β/IFNγ-activated DLD-1 colon carcinoma cells are the tumor suppressive molecules IP-10 and MIG. In contrast to IL-8, expression of these two mediators required cellular activation by IFNγ. Moreover, a coincubation with IL1β/IFNγ was necessary to detect IP-10 production after an 8h incubation period (Fig.15A). The observed strong synergistic effect between IL-1β and IFNγ concerning IP-10 release and a 8h lag period in cells stimulated with IFNγ alone (Fig.15A) agrees with recent data on human visceral epithelial cells (glomerular podocytes) (Romagnani P., et al. 2002). Preincubation of DLD-1 cells with DETA-NO potently reduced expression of IP-10 (Fig.15B) and MIG (Fig.15C). In addition, we also investigated regulation of iNOS by NO. Like IP-10 and MIG, iNOS is an IFNγ-dependent gene in DLD-1 cells (Fig. 12). As shown in Fig. 16, preincubation with NO reduced IL- 1 β/IFNγ-mediated mRNA (A) and protein expression of iNOS (B). This observation contrasts with regulatory actions of NO on iNOS expression in renal mesangial cells. In this cellular system iNOS is inducible by IL-1β alone. Similar to data on IL-1β-dependent IL-8 regulation in DLD-1 cells, NO enhanced IL-1β-induced iNOS expression in mesangial cells (Fig.16C), which corrobates previous data (Mühl H., and Pfeilschifter J., 1995).



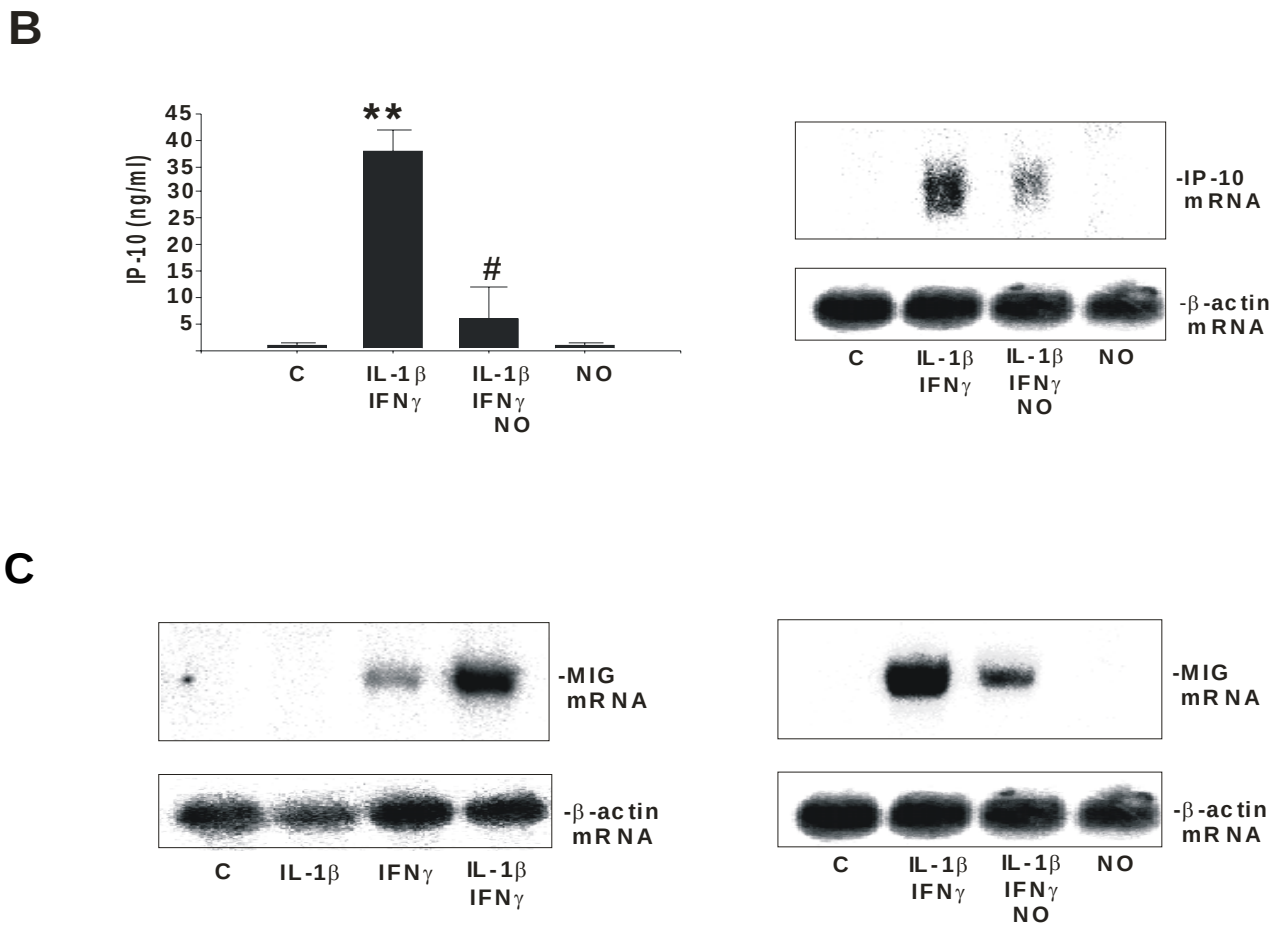


Fig. 15: (A-C) NO suppresses IL-1β/IFNγ-induced IP-10 and MIG expression. (A) DLD-1 cells were kept as unstimulated control or incubated with IL-1β (50 ng/ml), with IFNγ (10 ng/ml), or with IL-1β/IFNγ. After 8h, release of IP-10 protein (left panel) and expression of IP-10 mRNA (right panel) were determined by ELISA and Northern blot analysis, respectively. Protein levels are expressed as mean IP-10 concentrations ± SD (n = 4). **, $p < 0.01$ compared to unstimulated control. One representative of three independently performed Northern blot experiments is shown. (B) DLD-1 cells were incubated as unstimulated control or with DETA-NO (1 mM). After 16h, IL-1β (50 ng/ml)/IFNγ (10 ng/ml) was added as indicated. Following an additional 8h incubation period, release of IP-10 protein (left panel) and expression of IP-10 mRNA (right panel) were determined by ELISA and Northern-blot analysis, respectively. Protein levels are expressed as mean IP-10 concentrations ± SD (n = 6). **, $p < 0.01$ compared to unstimulated control; #, $p < 0.01$ compared to IL-lβ/IFNγ alone. One representative of three independently performed Northern blot experiments is shown. (C, left panel) DLD- 1 cells were kept as unstimulated control or incubated with IL- 1β (50 ng/ml), with IFNγ (10 ng/ml), and with IL-Iβ/IFNγ. After 8h, expression of MIG mRNA was determined by Northern-blot analysis. One representative of three independently performed Northern-blot experiments is shown. (C, right panel) DLD-1 cells were incubated as unstimulated control or with DETA-NO (1 mM). After 16h, IL- 1β (50 ng/ml)/IFNg (10 ng/ml) was added as indicated. Following an additional 8h incubation period,

expression of MIG mRNA was detennined by Northern-blot analysis. One representative of three independently performed Northern-blot experiments is shown.

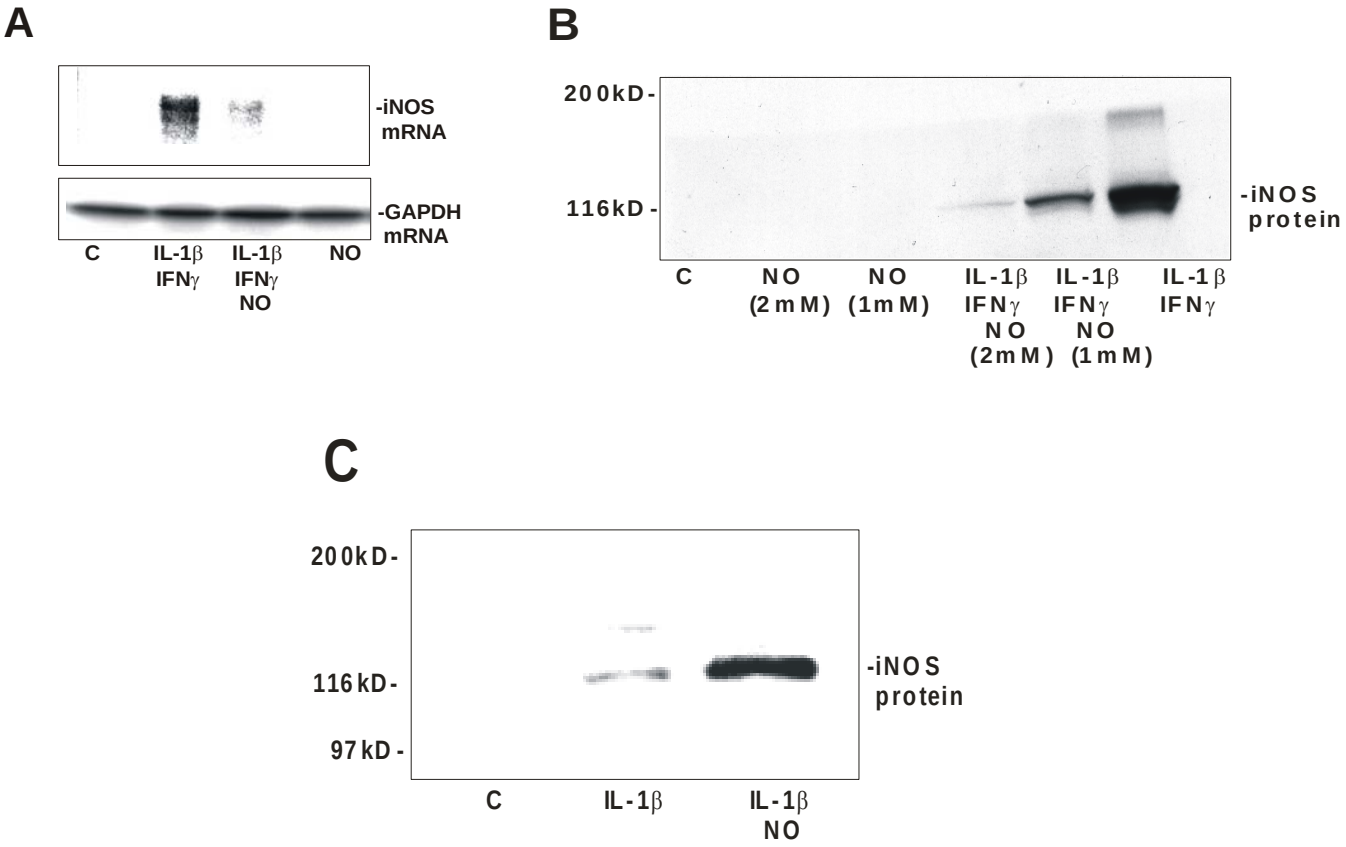


Fig. 16: (A-C) NO differentially regulates IFNγ- and IL-1β-driven expression of iNOS. DLD-1 cells were incubated as unstimulated control or with DETA-NO (NO, 1 mM). After 16h, IL-1β (50 ng/ml)/IFNγ (10 ng/ml) was added. Following an additional 24h incubation period expression of iNOS was determined by RNase protection assay (A) and immunoblot analysis (B), respectively. One representative of three independently performed RNase protection assays and immunoblot experiments are shown. (C) Rat renal mesangial cells were kept as unstimulated control, or stimulated for 24h with IL-1β (50 ng/ml) alone, or in combination with DETA-NO (NO, 1 mM). Thereafter, iNOS expression was investigated by immunoblot analysis (C).

4 Discussion

4.1 Aim of the Study

We investigated the expression of parameters of angiogenesis in activated DLD-1 colon carcinoma cells. In order to activate these cells two stimulatory signals were used:
In the first part of the study cells were exposed to PDTC which is an AP-1 activating agent that has been proposed as a treatment option of colorectal cancer in combination with chemotherapeutic drugs.
In the second part we studied effects of NO which is an immunmodulatory radical that is endogenously produced in the microenvironment of human colon cancer.

4.2 The role of chemokines in cancer

Cancer is a devastating disease for which standard therapy frequently fails. An estimated 5.2 million people die each year from cancer-related deaths around the world. Novel therapies that gained popularity because of recent advances are anti-angiogenesis drugs and tumor vaccines (Parkin, D.M, et al. 1999). Chemokines are particularly promising molecules because they have proved useful as both anti-angiogenic agents and critical determinants for successful tumor vaccine development in preclinical animal models (Belperio, J.A. et al. 2000; Fushimi, T. et al. 2000; Sharma, S. et al. 2000) .
Chemokines, as with other cytokines, are meant to function locally, and systemic delivery could result in unwanted side effects and toxicity. One promising avenue is the delivery of chemokines by conventional gene therapy vectors, which has been successfully employed by several investigators in murine models (Addison, C.L. et al. 2000; Fushimi, T. et al. 2000; Narvaiza, I. et al. 2000; Emtage, P.C. et al. 1999). An advantage of using chemokines is that, as opposed to tumor suppressor or death genes, not necessarily every tumor cell has to be transduced. Thus, if a proportion of tumor cells secrete an angiostatic molecule, the balance of angiogenic factors in the tumor environment might be altered sufficiently to prevent neovascularisation and further cancer growth. This concept is even more apparent for the development of tumor vaccines, which initially requires interaction between only a subset of tumor cells, antigen-presenting cells and T cells.

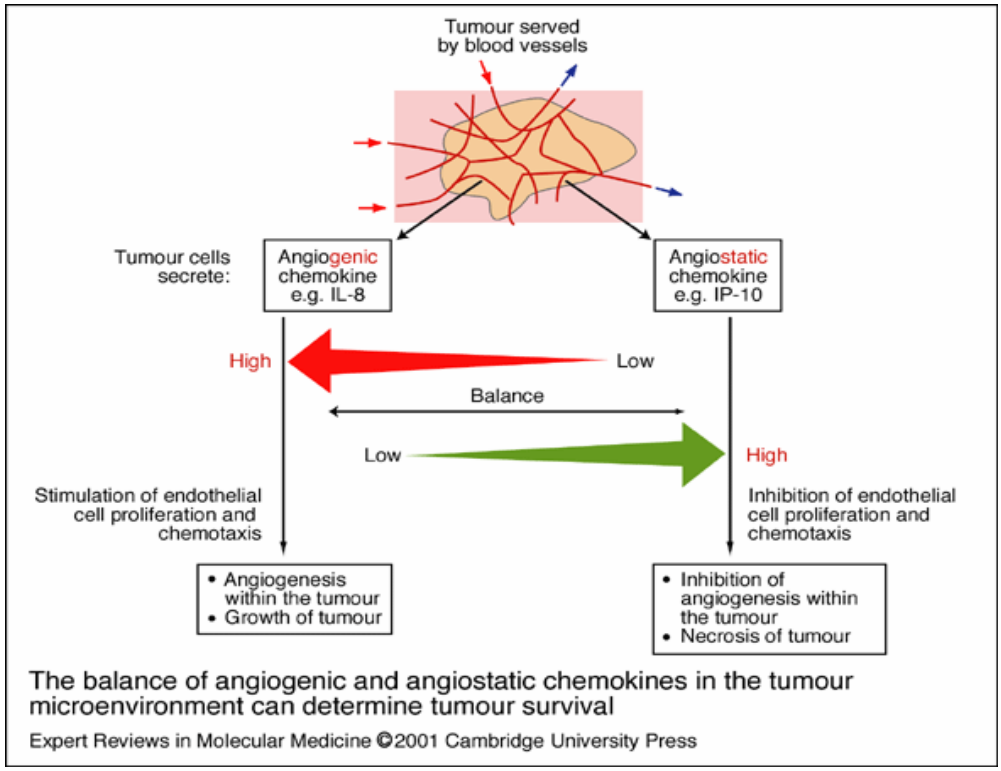


Figure 17: The balance of angiogenic and angiostatic chemokines in the tumor microenvironment can determine tumor survival. Chemokines can stimulate or inhibit proliferation and chemotaxis of endothelial cells of the blood vessels that serve a tumor. If a tumor secretes excess of angiogenic chemokines [e.g. interleukin 8 (IL-8)] angiogenesis is stimulated, leading to new blood vessel formation and continued tumor growth. An excess of angiostatic chemokines [e.g. interferon-γ-inducible protein 10 (IP-10)] in the tumor microenvironment inhibits neovascularisation, leading to tumor necrosis and regression (adapted from Mitchell J. Frederick and Gary L. Clayman, 2001).

4.2.1 IL-8, VEGF and HO-1 in angiogenesis

IL-8 and VEGF are two mediators of angiogenesis and tumor progression which are coexpressed in a variety of human tumors, including colorectal cancer (Fox, S.H., 1998; Bancroft, C.C.,2001). IL-8 was the first chemokine discovered to stimulate endothelial cell chemotaxis, proliferation and angiogenesis *in vivo* (Koch, A.E. et al. 1992). Many tumors overexpress IL-8, compared with their surrounding non-malignant tissue. IL-8 is an autocrine growth factor for certain melanomas (Schadendorf, D. et al. 1993) as well as tumor cell lines derived from cancers of the colon, stomach, liver, pancreas and skin (Fujisawa, N. et al. 2000; Brew, R. et al. 2000; Miyamoto, M. et al. 1998; Metzner, B. et al. 1999). In biopsy tissue from ovarian carcinomas, neuroblastomas and squamous cell carcinomas of the head and neck, both IL-8 and its receptor are expressed by cancerous cells, suggesting that IL-8 could also function in an autocrine factor for these tumors (Ivarsson, K. et al. 2000; Ferrer, F.A. et al. 2000; Richards, B.L. et al. 1997). The autocrine function of IL-8 illustrates the pleiotropism of chemokines, as these particular molecules not only stimulate tumors to multiply, but also recruit endothelial cells in vivo to ensure that

tumors develop an adequate vasculature as they grow. IL-8 is perhaps one of the most ideal tumor growth factors (Mitchell J.F., et al. 2001). In many cell types the synthesis of IL-8 is strongly induced by IL-1β and/or TNF-α. IL-8, IL-1β, and TNF-α are known collectively as major proinflammatory cytokines because they accelerate inflammation and also regulate inflammatory reactions either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in certain cell types. The net effect of an inflammatory response is determined by the balance between proinflammatory cytokines and antiinflammatory cytokines (see also introduction). IL-8 is constitutively produced by various carcinoma cells and this synthesis may be related to the elevation of serum IL-8 in patients with hepatocellular carcinoma (Zwahlen, R., et al. 1993).

One of the most important growth and survival factors for the endothelium is vascular endothelial growth factor (VEGF) (Ferrara, N. et al. 1999). VEGF induces angiogenesis and endothelial cell proliferation and plays an important role in regulating vasculogenesis. VEGF is a heparin-binding glycoprotein that is secreted as a homodimer (Houck, K.A. *et al.* 1992; Park, J.E. *et al.* 1993; Houck, K.A. *et al.* 1991). Most types of cells, but usually not endothelial cells themselves, secrete VEGF. VEGF has been renamed to VEGF-A, following the identification of several VEGF-related factors (VEGF-B, VEGF-C, VEGF-D, VEGF-E). VEGF is a homodimeric heavily glycosylated protein of 46-48 kDa (24 kDa subunits). However, glycosylation is not required, for biological activity. The subunits of VEGF are linked by disulphide bonds. The human factor occurs in several molecular variants of 121 (VEGF-121), 165 (VEGF-165), 183 (VEGF-183), 189 (VEGF-189) and 206 (VEGF-206) amino acids, arising by alternative splicing of the mRNA. Another variant has been designated VEGF-C . Other VEGF-related factors are e.g. VEGF-B , which forms heterodimers with VEGF-A, and VEGF-C . Since it is a potent regulator of vascular permability, VEGF was formaly also known as vascular permability factor (Dvorak, H.F. *et al.* 1999). In addition, VEGF causes vasodilatation, partly through stimulation of constitutive nitric oxide synthase in endothelial cells (Yang, R. *et al.* 1996). VEGF can also stimulate cell migration and inhibit apoptosis (Alon, T. *et al.* 1995). Several splice variants of VEGF-A have heparin-binding domains, which help anchor them in the extracellular matrix and facilitate presentation to VEGF receptors. VEGF-A transcription is potentiated in response to hypoxia. The transcription factors which are involved are hypoxia inducible factor-1α (HIF-1α) and HIF-2α. These are

degraded by the proteasome pathway in normoxia but stabilized in hypoxia (Kallio, P.J. *et al.* 1999). HIF-1α and HIF-2 α heterodimerize with the aryl hydrocarbon nuclear translocator (ARNT) in the nucleus and bind the VEGF promoter/enhancer. This is a key pathway operating in most types of cells. VEGF transcription in normoxia is activated by many oncogenes including H-ras and several transmembrane tyrosine kinases such as the epidermal growth factor receptor and erbB2 (Arbiser, J.L. *et al.* 1997; Okada, F. *et al.* 1998; Petit, A.M. *et al.* 1997). Together, hypoxia and oncogenic activation, may account for a marked upregulation of VEGF-A in tumors compared to normal tissues, a process that is of prognostic relevance in clinical practice (Gaspeirini, F. *et al.* 1997). VEGF can be detected in serum samples of cancer patients (Banks, R.E. *et al.* 1998). Platelets release VEGF upon aggregation and may be a major source of VEGF delivery to tumors (Pinedo, H.M. *et al.* (1998). Several studies have shown that high serum levels of VEGF and poor prognosis in cancer patients may be correlated with an elevated platelet count (O'Byrne, K.J. *et al.* 1999). Many tumors release cytokines that can stimulate the production of megakaryocytes in the bone marrow and elevate the platelet count. This can result in an indirect increase of VEGF delivery to tumors (Salgado, R. *et al.* 1999). Besides tumorgenesis VEGF is implicated in several other pathological conditions associated with enhanced angiogenesis. For example, VEGF plays a role in psoriasis, diabetic retinopathy, and rheumatoid arthritis (Koch, A.E. *et al.* 1994; Ferrara, N. *et al.* 1998). Direct demonstration of the importance of VEGF in tumor growth has been achieved by using dominant negative VEGF receptors (Millauer, B. *et al.* 1996) and neutralizing antibodies for VEGF (Witte, L. *et al.* 1998) or VEGF receptors. Interference with VEGF function has therefore become of major interest for drug development with the aim to block angiogenesis. These approaches include antagonists of VEGF or its receptors, selective VEGF receptor tyrosine kinase inhibitors (Piossek C., *et al.* 1999), targeting of drugs and toxins to VEGF receptors (Saleh, M. *et al.* 1996), as well as hypoxia driven gene therapy targeting VEGF or the VEGF signaling pathway (Dachs G.U., *et al,* 1997).

HO-1 is a 32-kDa protein that is inducible by numerous stimuli. HO-1 catalyzes the first and rate-limiting step in the degradation of heme. Via oxidation, HO-1 cleaves the α-meso carbon bridge of β-type heme molecules to yield equimolar quantities of biliverdin IXa, CO, and free iron. Biliverdin is subsequently converted to bilirubin via the action of biliverdin reductase, and free iron is promptly sequestered into ferritin.

To date, three isoforms (HO-1, HO-2, and HO-3) that catalyze this reaction have been identified (Abraham, N.G., et al. 1987; Maines, M.D. 1988; Mc Coubrey, W.K., et al. 1992; Mc Coubrey, W.K., et al. 1997). Although heme is the typical HO-1 inducer, several studies demonstrated that HO enzyme activity could also be stimulated by a variety of nonheme products including ultraviolett irradiation, LPS, heavy metals, NO and oxidants such as hydrogen peroxide (Keyse and Tyrell, 1987; Maeshima et al., 1996; Vile and Tyrell, 1993). One common feature of these inducers is their capacity to generate reactive oxygen species. HO-1 can function as a cytoprotective molecule against oxidative stress. Indeed, ample evidence currently supports the notion that HO-1 serves to provide potent cytoprotective and antiinflammatory effects in many *in vitro* and *in vivo* models of oxidant-induced cellular and tissue injury.
As for IL-8 and VEGF, induction and activity of HO-1 has been associated with growth of solid tumors (Doi, K., et al. 1999 ; Nishie, A., et al. 1999).

4.3 The role of PDTC as drug in clinical trials and its mode of action

Primarily due to its capability of inducing apoptosis in certain cancer cells, use of PDTC as an anticancer drug has been proposed (Chinery, R., 1997; Bach, S.P.2000; Della Ragione, F. 2000). PDTC is a member of the dithiocarbamate family, well known for its ability to bind free or protein-bound metal (Orrenius, S., et al. 1996). Dithiocarbamates exert both antioxidant and prooxidant effects in cells. Their antioxidant behaviour includes eliminating hydrogen peroxide (Mankhetkorn, S., et al. 1994) and the scavenging of superoxide radicals (Mankhetkorn, S., et al. 1994), peroxynitrite (Liu, J., et al. 1996), hydroxyl radicals (Liu, J., et al. 1996), as well as the peroxyl radicals (Bartoli, G. M., et al. 1993; Zanocco, A. L., et al. 1989). The reaction of dithiocarbamates with reactive oxygen and nitrogen species generates dithiocarbamate thiyl radicals which ultimately dimerize to form thiuram disulphides (Mankhetkorn, S., et al. 1994; Liu, J., et al. 1996; Zanocco, A. L., et al. 1989), the oxidized form of dithiocarbamates. Thiuram disulphides are responsible for much of the prooxidant characteristics of dithiocarbamates. PDTC thus can potently oxidize GSH (reduced glutathione) a cellular protectant, and protein thiols (Orrenius, S., et al. 1996; Hosni, M. et al. 1992; Nobel, C. S. I., et al. 1995). In the analysis of

dithiocarbamate action, the antioxidant behaviour of these agents has been more often acknowledged, with less appreciation for their prooxidant character. For example, although inhibition of NFκB activation by PDTC has often been attributed to its radical-scavenging properties (Schreck, R., et al 1992; Meyer, M., et al. 1993), it has recently been demonstrated that PDTC may exert its inhibitory effect on NFκB via direct oxidation of critical thiols located in this transcription factor (Brennan, P. and O'Neill, L. A. J. 1995; Brennan, P. and O'Neill, L. A. J. 1996). Recent evidence suggests, that the reactive oxygen species (ROS) model of NFκB activation may be restricted to certain cell types and that this activation pathway may not be required for the stimulation of NFκB by IL1-β and TNFα. The prooxidant consequences of dithiocarbamate action, have recently been highlighted with respect to their infuence on apoptosis (Orrenius, S., et al. 1996; Nobel, C. S. I., 1997). PDTC has become more and more of interest in relation to cancer therapy particularly in combination with classical cytostatic agents. In preclinical studies (Chinery, R., et al. 1997), antioxidants have been tested as potential therapeutic agents for treatment of colorectal cancer. In these studies, it has been found that PDTC can induce growth arrest, activation of $p21^{WAF/CIP1}$ (WAF1 denotes wild-type p53-activated fragment-1 and CIP1 denotes cyclin-dependent kinase-interacting protein-1) and apoptosis in colorectal cancer cells *in vitro*. Moreover, PDTC can induce regression of human colon cancer tumors in nude mice when given in combination with 5-FU (Beauchamp,R.D., et al 1996). Actions of PDTC on cells also include potent activation of the transcription factor AP-1 (Yokoo, et al 1995), which is involved in cellular proliferation, transformation, and death (Shaulian and Karin, 2002). PDTC enhances the DNA-binding activity of AP-1, as well as c-fos and c-jun mRNA levels (Munoz C. et al. 1996). AP-1 is not a single protein, they belong to a group of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun/Fos subfamilies. The growth-promoting activity of AP-1 is mediated by c-Jun. c-Jun mediates repression of tumor suppressors, as well as upregulation of positive cell cycle regulators. Mostly, c-Jun is a positive regulator of cell proliferation, whereas JunB has the converse effect (Shaulian and Karin, 2002). The induction of AP-1 by proinflammatory cytokines and stressors like PDTC is mostly mediated by phosphorylation of tyrosine kinases (Yokoo T. and Kitamura M. 1996).

4.4 Effects of PDTC on the expression of IL-8, VEGF, and HO-1

We investigated effects of PDTC on the expression of IL-8, VEGF, and HO-1. In this work we report that expression of this particular set of genes is induced by PDTC in human DLD-1 colon carcinoma cells. In contrast, treatment with the antioxidant acetylcysteine did not mediate IL-8 expression. Induction of HO-1 by PDTC agrees with recent data from rat aortic smooth muscle cells (Hartsfield, C.L., et al, 1998). In addition, we demonstrate that PDTC can efficiently enhance TNFα-mediated release of IL-8 from DLD-1 cells. At first sight these observations appear unexpected as IL-8 is an NF-κB dependent gene (Roebuck, K.A. 1999) and NF-κB activation can be inhibited significantly by treatment with PDTC in certain cell types (Schreck, R., et al. 1992; Eberhardt, W., et al. 1994). However, in human colon carcinoma cells inhibition of NF-κB activation by PDTC was not observed (Chinery, R., et al. 1997; Hellin, A.C., et al 1998). In leukemic promonocytic U937 cells, PDTC not only was unable to inhibit TNFα-induced NF-κB activation, but actually enhanced TNFα-mediated κB-dependent gene induction (Watanabe, K., et al. 1999). Moreover, TNFα-induced NF-κB binding activity could only be partially suppressed (50% inhibition) by PDTC at 100 μM in SW620 human colon carcinoma cells (Wahl, C., et al. 1998). In keeping with previous reports, we confirm constitutive NF-κB binding activity in human colon carcinoma cells as seen in DLD-1 cells (see also results Fig.4B; Kleinert, H., et al. 1998) or SW48 cells (Lind, D.S., et al. 2001). This constitutive activity was not reduced by treatment with PDTC in DLD-1cells. Molecular cooperation particularly between the transcription factors NF-κB and AP-1 has been identified as an essential prerequisite for optimal gene induction of IL-8 in epithelial cells (Mastronarde, J.G., et al. 1998). As already mentioned PDTC is a well characterized activator of AP-1 (Munoz, C., et al. 1996; Aragones, J.,et al. 1996; Yokoo, T. and Kitamura, M. 1996; Pinkus, R., et al. 1996; Hartsfield, C.L.,et al. 1998; Meyer, M., et al. 1993). In the present study we were able to confirm activation of AP-1 by PDTC using EMSA analysis. We investigated also the importance of the binding sites for AP-1 and NF-κB with regard to PDTC-induced IL-8 by mutational analysis of the IL-8 promotor. The NF-κB- (-80 to -70 nt) as well as the AP-1-binding site (-127 to -120 nt) (Roebuck, K.A. (1999) both turned out to be necessary for PDTC-induced IL-8.

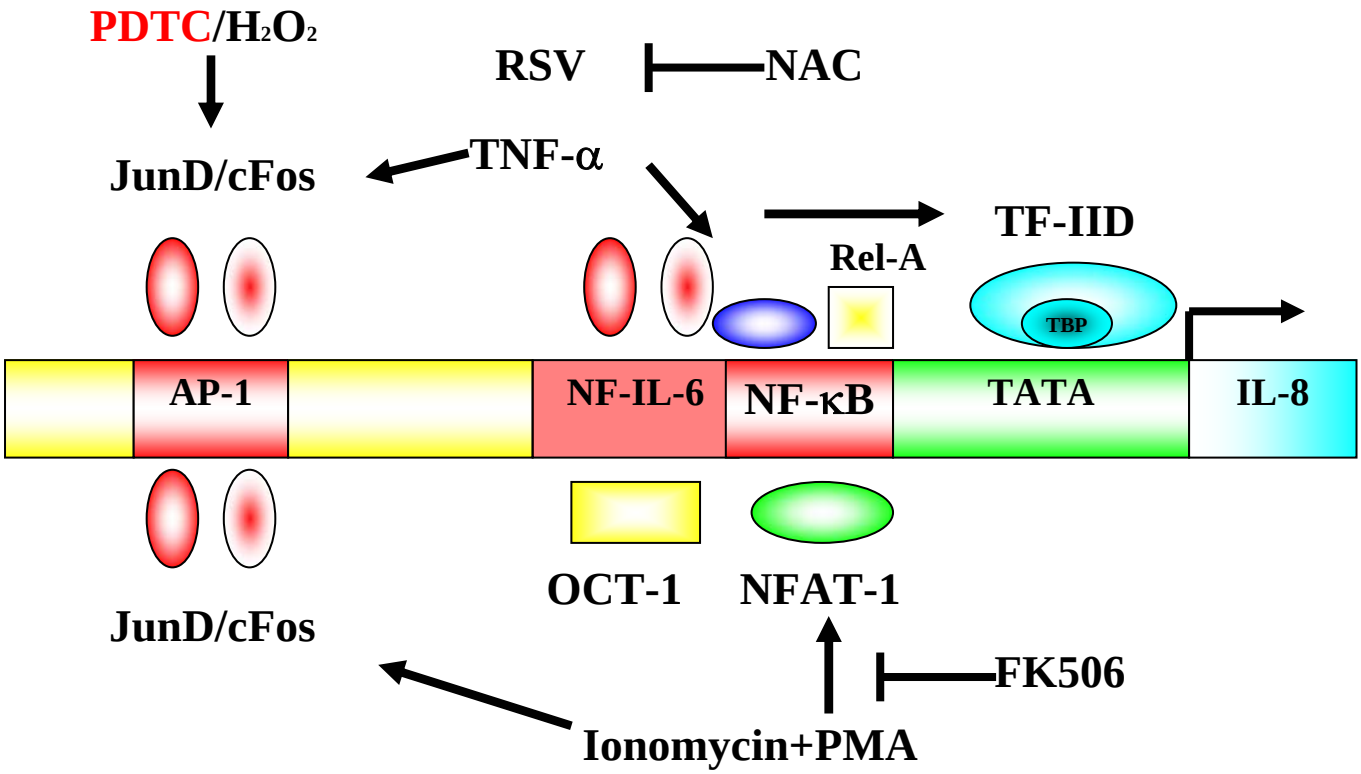


Figure 18: Schematic arrangement of putative transcription factor binding sites in the IL-8 promotor

These data imply that PDTC-induced AP-1 cooperates with constitutive NF-κB activity in DLD-1 cells which then drive synergistic expression of IL-8. In addition, mutational analysis of the C/EBPβ binding site (-94 to –81 nt) suggested that PDTC-induced activation of C/EBPβ (Chinery, R., et al. 1997) contributes to a certain degree to IL-8 promotor activation by this agent. This observation is in keeping with previous reports demonstrating the capability of C/EBPβ to enhance NF-κB dependent IL-8 expression (Stein, B. and Baldwin, A.S. Jr. 1993).

Induction of IL-8 by PDTC was not restricted to colon carcinoma cells but was also detectable in human PBMC, where monocytes are regarded as the major source of IL-8 (DeForge, L.E., et al. 1992). Accordingly, PDTC has been shown previously to activate AP-1 in monocytic cells (Watanabe, K., et al. 1999). In the present study, PBMC were isolated from whole blood by density separation over a Ficoll-Hypaque gradient, which is standard methodology. This procedure is unevitably associated with activation of PBMC, as shown by induction of TNFα mRNA expression (Hartel,

C., et al. 2001), a process likely to be mediated by activation of NF-κB. Therefore, upregulation of IL-8 in PBMC is in keeping with the hypothesis that PDTC-stimulated AP-1 mediates IL-8 production in preactivated cells. Augmentation of IL-8 release from PBMC by PDTC was however modest compared to inflammatory stimuli such as IL-1β or LPS (Fig.5). The present observation that IL-8 can be induced by PDTC as a single stimulus appears to be of particular significance in the context of transformed colon carcinoma cells in which NF-κB is not (Chinery, R., et al. 1997; Hellin, A.C., et al. 1998; present data) or only partially (Wahl, C., et al. 1998) modulated by PDTC. Thus our data do not necessarily contradict the antiinflammatory potential of PDTC (Lauzurica, P., et al.1999; Munoz, C., et al. 1996).

Lately it has been reported that IL-1β-induced IL-8 secretion in the human intestinal cell line HT-29 could be attenuated by PDTC in a dose dependent manner (Nemeth ZH, et al. 2003). Furthermore, accumulation of IL-8 mRNA was suppressed. In these cells activation of IL-8 was inhibited because IL-1β-induced NFκB DNA binding and NFκB-dependent transcriptional activity was potently suppressed by PDTC. In contrary to DLD-1 cells, HT-29 cells have a very low basal level of NFκB activation. Thus, the status of NFκB in DLD-1 cells is quite different from that in HT-29 cells. Again, in contrast to the situation in DLD-1 cells, NFκB binding was potently upregulated by IL-1β in HT-29 cells and this activation was inhibited by PDTC. Consequently, subsequent IL-1β-induced IL-8 was inhibited by PDTC in these cells. In conclusion, these results from the HT-29 study concure with our concept that the state of cellular NFκB activation in a given cell type determines PDTC actions on these cells. In DLD-1 cells PDTC-induced activation of AP-1 synergizes with constitutive NFκB for IL-8 production. In HT-29 cells, NFκB is not constitutively activated and PDTC-induced AP-1 alone is not sufficient to trigger IL-8 expression.

We did not further investigate the molecular basis of PDTC-induced VEGF and HO-1 gene activation. However, similar to IL-8, expression of VEGF (Bancroft, C.C., et al. 2001; Damert, A., et al. 1997) and HO-1 (Alam, J. and Den, Z. 1992) can be induced by activation of AP-1 in cancer cells. AP-1 in particular mediates PDTC-induced HO-1 gene induction in the murine transformed macrophage-like cell line RAW 264.7 (Hartsfield, C.L., et al. 1998).

Recently, it has been reported that NF-κB binding activity is augmented in human colorectal cancer (Lind, D.S., et al. 2001). The present data imply that exposure of these tumor tissues to PDTC during chemotherapeutic intervention may mediate expression of proangiogenic IL-8 and VEGF, as well as HO-1 in colon carcinoma cells. A cellular response that could adversely affect the efficacy of PDTC in cancer therapy.

4.5 Effects of NO on the expression of IL-8, VEGF, MIG, and IP-10

Upregulation of iNOS protein and activity has been observed in a variety of human cancers e.g. colorectal cancer, breast cancer (Thomsen, L.L., et al. 1995), in tumors of the central nervous system (Cobbs, C.S.,et al.1995), in prostate cancer (Klotz, T., et al. 1998), in lung cancer (Liu, C.Y., et al. 1998), in ovarian cancer (Thomsen, L.L.,et al. 1998), as well as in melanomas (Tschugguel, W., et al.1999), and head and neck cancer (Gallo 0., et al. 1998). Cell culture experiments suggest an antiproliferative role of NO in colon carcinoma cells (Buga, G.M., et al 1998). Furthermore, apoptosis of cancer cells driven by high levels of NO is a well described phenomenon (Cui S., et al. 1994). However, analysis of iNOS expression in human tumor tissues and murine tumor models may point to a different role of NO in tumor biology. It has been shown that expression of iNOS in human colon cancer tissues is positively correlated with the frequency of mutations in the p53 tumor suppressor gene. This implies that NO provides a significant selection pressure for nonfunctional p53, a mechanism that may ultimately promote tumor growth (Ambs, S., et al. 1999). It has also been reported that compared to wild-type cells, overexpression of iNOS in DLD-1 cells results in enhanced tumor growth when these cells are injected into nude mice. Tumors derived from these iNOS overexpressing carcinoma cells appear markedly more vascularized (Jenkins, D.C., et al 1995). Similar observations have been reported using Calu-6 lung carcinoma cells (Ambs, S., et al. 1998). Notably, DLD-1 cells (Kagawa, S., et al. 1997) and Calu-6 (Ambs, S., et al. 1998) cells do not express wild-type p53 since NO can mediate apoptosis via p53 (Messmer U., et al, 1994) This lack of wild-type p53 appears to be an important determinant of NO action in these murine models of tumor growth (Ambs, S., et al. 1998). NO-mediated tumor promotion agrees with a report investigating expression of iNOS in human head and

neck cancer (Gallo, 0., et al. 1998). In that study expression of iNOS is proposed as a marker of tumor progression which closely correlates with angiogenesis and tumor vascularization. A similar association of iNOS, angiogenesis and tumor progression has been observed in a model of mammary cancer (Jadeski, L.C., and Lala, P.K. 1999). As tumor angiogenesis is recognized as a chief parameter determining tumor growth (Kerbel, R.S. 2000) , we investigated effects of NO on DLD-1 cells with respect to expression of four well documented regulators of angiogenesis, namely angiogenic VEGF (Holash, J., et al. 1999) and IL-8 (Koch, A.E., et al. 1992) as well as angiostatic IP-10 (Luster, A.D., et al. 1995) and MIG (Addison C.L., et al. 2000). Here, we report that IL-1β-dependent production of IL-8 in DLD-1 cells is significantly increased by coincubation with NO. Upregulation of IL-8 release by NO agrees with reports on IL-8 regulation in human mesangial cells (Brown, Z., et al. 1993), endothelial cells (Villarete L.H., and Remick D.G. (1995), keratinocytes (Wetzler C., et al. 2000), and monocytes (Mühl H., et al. 2000). It is interesting to note that IL-8 not only is as active as VEGF in mediating angiogenesis (Koch, A.E., et al. 1992), but is also a growth factor for colon carcinoma cells. (Brew R., et al. 2000; Li A., et al. 2001). In accord with previous data on glioblastoma cells and hepatocellular carcinoma cells (Chin, K., et al. 1997), constitutive production of VEGF in DLD-1 cells was enhanced by NO. Likewise, overexpression of iNOS can mediate amplification of VEGF production (Jozkowicz A., et al. 2001)). Moreover, reduced VEGF has been detected in tumors of iNOS deficient mice (Kisley L.R., et al. 2002). Contrary to these promotors of tumor growth, we observed that expression of the angiostatic chemokines IP-10 and MIG is efficiently suppressed under the influence of NO. A similar NO-induced downregulation of IP-10 and MIG expression has been reported in human vascular endothelial cells and visceral epithelial cells (glomerular podocytes) (Mach, F., et al. 1999), Thomsen, L.L., et al. 1995).
Suppression of IP-10 and MIG is likely a key event that is mediated by NO in the tumor microenvironment. Both CXC chemokines bind to the same receptor (CXCR3) and both are often coexpressed as shown for EBV-positive lymphomatoid granulomatosis and nasal-type T cell/NK cell lymphomas. In these cancerous tissues, high levels of MIG and IP-10 are associated with necrosis and vascular damage (Teruya Feldstein J. et al, 1997). IP-10 and MIG are chemoattractant for activated T cells. Actually, strong expression of MIG has been detected in human malignant melanomas in areas of heavy T cell infiltration. Evidence suggests that MIG is critical

for control of melanoma growth (Kunz M., et al. 1999). The relevance of these two chemokines is further underscored by the observation that injection of MIG into Burkitt tumors grown in nude mice causes tumor necrosis and extensive vascular damage (Sgadari C., et al, 1997). Similar antitumor effects have been observed by use of tumor cells genetically engineered to express IP-10 (Angiolillo A.L., et al, 1995).

As previously described for DLD1 cells, IFNγ-dependent induction of iNOS was similarly impaired by pretreatment with NO (Cavicchl M., et al. 2000). Supression of iNOS expression should serve to control overproduction of NO that may be deleterious. However, effects of NO on iNOS protein appear to be species and stimulus dependent, as expression of this enzyme in response to IL-1β is potently enhanced by NO in rat mesangial cells or smooth muscle cells (Fig.5c) (Mühl H., et al. 1995; Boese M., et al.1996). These results suggest that NO may be able to specifically downregulate cellular responses to IFNγ in DLD-1 cells. This notion concures with a report that demonstrates the capability of NO to interfere with activation of STAT-1 (Llovera M., et al. 2001), a pivotal component of IFNγ signaling. Taken together, NO differentially regulated expression of modulators of angiogenesis in DLD-1 colon carcinoma cells. Production of tumor promoting IL-8 and VEGF was amplified, whereas in the same cells expression of tumorstatic IP-10 and MIG was efficiently downregulated by NO. IP-10 and MIG are supposed to be key mediators of IFNγ mediated tumor regression (Belperio, J.A., et al. 2000). The regulatory function of NO may contribute to the phenomenon of tumor promotion by NO. The present data are in accord with reports on control of colon carcinogenesis in rodents by blockage of iNOS activity (Kisley L.R., et al. 2002; Rao C.V., et al. 1999) and further support the concept of iNOS inhibition as a therapeutic strategy for the treatment of human cancer.

5 Summary

Interleukin (IL)-8, heme oxygenase-1 (HO-1), and vascular endothelial growth factor (VEGF) appear to be critically involved in immune responses associated with inflammation, infection, and tumor growth. Regulation of these mediators was studied in the human colon carcinoma cell line DLD-1. Here we report that pyrrolidine dithiocarbamate (PDTC) not only augmented tumor necrosis factor-α induced release of IL-8, but also mediated IL-8 expression as a single stimulus. Mutational analysis of the IL-8 promotor and electrophoretic mobility shift analysis revealed that activation of the transcription factor activator protein-1 (AP-1) and a constitutive nuclear factor-κB (NF-κB) binding activity in DLD-1 cells were mandatory for PDTC-induced IL-8 expression. Besides IL-8, PDTC also upregulated expression of HO-1 and VEGF in these cells. Induction of IL-8 by PDTC was not restricted to DLD-1 cells, but was observed as well in Caco-2 colon carcinoma cells and in peripheral blood mononuclear cells. PDTC is currently advocated for use as chemotherapeutic drug in the treatment of certain malignancies, among them colorectal cancer. Induction of IL-8, HO-1, and VEGF may affect therapeutic applications of this agent.

Expression of inducible nitric oxide (NO) synthase and production of NO appears to be a marker of tumor progression in human neoplasia, among them melanoma, head and neck cancer, and colorectal cancer. Since tumor-promoting functions of NO have been associated with increased angiogenesis at the tumor site, we investigated effects of NO on the production of selected chemokines that are supposed to differentially regulate tumor growth, namely proangiogenic IL-8 as well as tumorsuppressive interferon-inducible protein-10 (IP-10) and monokine induced by interferon-γ (MIG). These chemokines are expressed by colon carcinoma cells after stimulation with the combination IL-1β/Interferon (IFN)γ. Under these conditions, release of IL-8 was exclusively mediated by IL-1β but not by IFNγ, whereas production of IP-10 and MIG was dependent on activation by IFNγ. Effects of NO were analyzed by incubation with the NO-donor DETA-NO. Expression and release of IL-8 from colon carcinoma cells was markedly upregulated by NO. In addition, NO enhanced gene expression of vascular endothelial growth factor (VEGF). Accordingly, basal release of VEGF was significantly augmented in cells exposed to NO. In contrast, IL-1β/IFNγ-induced production of IP-10 and MIG was suppressed by NO. Likewise, overproduction of IFNγ-dependent inducible NO synthase was

restrained by NO in DLD-1 cells. The present data are consistent with previous observations that relate NO to enhanced tumor angiogenesis and imply that NO-mediated upregulation of IL-8 and VEGF as well as downregulation of IP-10 and MIG expression may contribute to this phenomenon.

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7 Appendix

7.1 Abbreviations

Chemicals and bioactive compounds

AP-1	activator protein-1
BSA	bovine serum albumin
bZIP	basic region-leucine zipper
C/EBP	CCAAT/enhancer-binding protein
CIP1	cyclin-dependent kinase-interacting protein 1
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
EDRF	endothelium-derived relaxing factor
EDTA	ethylendiamin-N,N,N',N'-tetraacetat
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
eNOS	endothelial NOS
FCS	fetal calf serum
FU	fluro uracil
GSNO	S-nitroso-glutathione
HIF-1α	hypoxia inducible factor-1α
HO-1	heme oxygenase-1
IFNγ	interferon gamma
IκB	inhibitors of NF-κB
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	interferon gamma inducible protein-10
JNK	c-Jun N-terminal kinase
LB	Luria Bertani
LDH	lactat dehydrogenase
L-NAME	(L)-N^G-nitroarginine methylester
LPS	lipo-poly sacharide
MAPK	mitogen-activated protein kinase
MEKK	MAP kinase kinase kinase

MHC	histocompatibility complex class
MIG	monokine induced by interferon gamma
MIP	macrophage inflammatory protein
MKK	MAP kinase kinase
NF-κB	nuclear factor kappa B
NK	natural killer
NLS	nuclear localization signal
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthases
ONOO(-)	peroxinitrite
PBMC	peripheral mononuclear blood cells
PBS	phosphate buffered saline
PDTC	pyrrolidine dithiocarbamate
PmxB	polymyxin-B
PVDF	polyvinylidene fluoride
RHD	Rel homology domain
RLUs	relative light units
ROI	reactive oxigen intermediates
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SDS	sodium dodesyl sulfate
SLC	secondary lymphoid organ cytokine
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
Stat 1	signal transducer and activator of transcription 1
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
WAF1	wild-type p53-activated fragment 1

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Sommer 1991 Praktikum bei der Firma BCE (Björnsen Consulting Engeniering = Ingenieurbüro tätig in der Umwelttechnik)

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7.4 List of publikations

Parts of this thesis have been published before.

Posters:

A) “Expression and Release of IL-8 Induced by the Antioxidant Pyrrolidine Dithiocarbamate in Monocytic Cells”. Frühjahrstagung der Pharmakologen und Toxikologen in Mainz 2000.

B) “Pyrroldidine Dithiocarbamate Induces Expression of Interleukin-8 in DLD-1 Colon Carcinoma Cells: Role of AP-1 and NF-IL6”. Frühjahrstagung der Pharmakologen und Toxikologen in Mainz 2001.

C) ”Pyrroldidine Dithiocarbamate Induces Expression of Interleukin-8,Heme Oxygenase-1 and Vascular Endothelial Growth Factor in DLD-1 Colon Carcinoma Cells”. 11th International Congress of Immunology in Stockholm.

D) “Nitric oxide differentially regulates expression of proangiogenic IL-8 and VEGF and tumorsuppressive IP-10 and MIG in colon carcinoma cells”. 33rd Annual Meeting of the German Society of Immunology in Marburg and VIII. NO-Forum in Frankfurt am Main.

Publikations:

Mühl H, Chang J-H, Huwiler A, Bosmann M., Paulukat J., Ninic R., Nold M., Hellmuth, M., Pfeilschifter J, ”Nitric oxide augments release of chemokines from monocytic U937 cells: modulation by anti-inflammatory pathways”. Free Radic Biol Med. 2000 Nov 15;29(10):969-80.

Markus Hellmuth, Christian Wetzler, Marcel Nold,Jae-Hyung Chang, Stefan Frank, Josef Pfeilschifter and Heiko Mühl “Expression of interleukin-8, heme oxygenase-1 and vascular endothelial growth factor in DLD-1 colon carcinoma cells exposed to pyrrolidine dithiocarbamate”. Carcinogenesis. 2002 Aug;23(8):1273-9.

7.5 Die Regulation des Tumorwachstums in Dickdarmkrebszellen wird beeinflusst von Stress ausgelöst durch PDTC und NO (Deutsche Zusammenfassung)

7.5.1Einleitung

Krebs ist eine verheerende Krankheit mit weltweit etwa 5,2 Millionen Todesopfern pro Jahr. Bei dieser Krankheit versagen Standardtherapiemethoden häufig (Parkin, D.M, et al. 1999). Neue Therapieansätze sind antiangiogene Pharmaka und Immuntherapeutische Ansätze gegen Tumore. Die Entwicklung neuer Therapien beruht zum Teil auf in jüngster Zeit erworbenen Kenntnissen über Zytokine und Chemokine (Belperio, J.A. et al. 2000; Fushimi, T. et al. 2000; Sharma, S. et al. 2000).

7.5.1.1 Zytokine

Zytokine sind kleine Proteine, meist Glykoproteine mit einem Molekulargewicht von 8 bis 40 kD. Sie fungieren als biochemischer Botenstoff zwischen Zellen. Das Wort Zytokin leitet sich aus den griechischen Wörtern für Zelle und Kinese ab.
Die Zytokine sind im Organismus bei einer Reihe von Prozessen involviert. Dazu gehören: Regulation der Immunantwort, Zellwachstum/-differenzierung und die Reparatur/Wiederherstellung von Gewebe.
Ein einzelnes Zytokin kann eine Vielzahl von verschiedenen biologischen Aktivitäten auslösen. Die meisten Zytokine werden von Leukozyten synthetisiert und wirken auch auf diese. Dies ist der Grund, warum die Zytokine bis heute auch noch als Interleukine bezeichnet werden. Diese Namensgebung wiederspiegelt jedoch nicht die biologische Realität, da man heute weiß, daß mit Hilfe dieser Botenstoffe auch Signale zwischen anderen Zelltypen übertragen werden. Die Fähigkeit von Zytokinen auf verschiedene Zelltypen zu wirken und dabei verschiedene biologische Effekte hervorzurufen, nennt man auch Pleiotropismus.
Synthese und die Wirkungsweise von Zytokinen werden durch andere Zytokine wiederum beeinflusst. Einige besitzen die Fähigkeit, sich in ihrer Wirkung zu ergänzen oder zu verstärken; während andere sich in ihrer Wirkung gegenseitig abschwächen.

Des weiteren besitzen Zytokine die Eigenschaft, die Synthese weiterer Zytokine zu induzieren, wodurch eine „Zytokin-Signal-Kaskade“ in Gang gesetzt werden kann. Dies kann bedeuten, daß für die Übertragung von einem biologischen Effekt durch ein Zytokin mehrere Zytokine benötigt werden.

Diese Effekte werden durch spezifische membrangebundene Rezeptoren übertragen. Die Expression von Zytokinrezeptoren ist ein wichtiger regulatorischer Faktor in der Zytokinbiologie. Festzuhalten ist, die Sekretion eines Zytokines ist von kurzer Dauer und selbst limitierend. Für die Produktion eines Zytokins ist normalerweise eine transiente Induktion der *de novo* Gentranskription erforderlich.

Eine Induktion der Gentranskription kann ausgelöst werden durch Stress oder immunologische Signale.

Entsprechend ihrer Einflüsse auf entzündliche Prozesse kann man die Zytokine in pro- und antientzündliche Zytokine einteilen.

Im Gegensatz zu antientzündlichen Zytokinen können proentzündliche in manchen Fällen das Krankheitsbild verschlechtern. Die antientzündlichen Zytokine haben die Funktion, die Entzündung zu hemmen und den Heilungsprozess zu fördern.

In einem gesunden Organismus sind nur geringe Konzentrationen der proentzündlichen Zytokine zu finden. Werden sie jedoch in höheren Konzentrationen sezerniert, so wird von ihnen eine Reihe von Genprodukten induziert. Hierzu gehören die induzierbare Stickstoffmonoxid-Synthase (NOS) und das Interleukin-8 (IL-8).

Dies führt letztendlich zu den Symptomen einer Entzündung, zu denen Rötungen, Schwellungen, Schmerz, Funktionsverlust und Zerfall des Gewebes gehören (Dinarello C.A. and Moldawer L.L., 2000; Janeway C.A. and Travers P., 1997; Abbas A., 2000).

Proentzündliche Zytokine aktivieren eine Reihe von wichtigen Transkriptionsfaktoren; diese sind unter anderen NF-κB, AP-1 und NF-IL-6.

Ein besonders wichtiges Zytokin ist das Interferon-γ (IFN–γ). Dies ist ein homodimeres Glykoprotein mit pleiotropher immunologischer Funktion bei der zellvermittelten Immunität gegen intrazelluläre Mikroben. IFN–γ kann die antimikrobielle Wirkung von Makrophagen verstärken, indem die Synthese von reaktiven Sauerstoffzwischenprodukten und Stickoxiden stimuliert wird (Stark, G.R. et al, 1998; Bach E. et al, 1997).

Neben der bedeutenden Funktion bei der Immunabwehr hat das IFN–γ auch eine wichtige Funktion als tumorhemmendes Zytokin. Die Interferon-γ Signalkaskade führt

zur Apoptose und zur Expression von immunmodulierdenden Proteinen, wie dem Interferon induzierbares Protein-10 (IP-10), dem Monokin induziert durch Interferon-γ (MIG) und der iNOS, die in Zusammenarbeit mit T-Zellen Tumore zerstören können (Ikeda H., et al, 2002).

7.5.1.2 Chemokine

Chemokine sind sind kleine Proteine, meist Glykoproteine, mit einem Molekulargewicht von 8 bis 17 kD. Es sind Zytokine, welche chemotaktische Reaktionen von Zellen induzieren und die Angiogenese regulieren.
Durch die Wirkung von Chemokinen sind Leukocyten in der Lage, sehr schnell auf Infektionen zu reagieren. Die Interaktion von chemotaktisch wirksamen Substanzen mit Leukocyten initiiert eine Reihe von biochemischen und zellulären Ereignissen. Hierzu gehören im einzelnen, die Veränderung von Ionenströmungen, Veränderung der Zellformen, die Sekretion lysosomaler Enzyme sowie die Produktion von Superoxidradikalen und eine bessere Beweglichkeit der Zellen.
Die Chemokine können aufgrund ihrer molekularen Struktur in vier Gruppen eingeteilt werden. Dies sind die CXC, CX_3C, CC und C Chemokine. Diese Bezeichnung beruht auf einer hoch konservierten Struktur zweier nahe beeinander stehenden Aminosäuren (Cystein) in der Aminosäurekette (Dinarello C.A. and Moldawer L.L., 2000; Janeway C.A. and Travers P., 1997; Abbas A., 2000). Die spezifischen Effekte der Chemokine werden durch transmembranäre G-Protein gekoppelte Rezeptoren vermittelt (Murphy PM.,1994).
Ebenso, wie bei den Zytokinen, kann man die Chemokine aufgrund ihrer Funktion einordnen. Wie schon im Falle der Zytokine, wo eine Verschiebung der Konzentrationen der anti- zu den proentzündlichen Zytokinen das Krankheitsbild der Infektion beeinflusst, ist etwas ähnliches im Falle der Chemokine in Bezug auf die Angiogenese möglich.
Chemokine beeinflussen den Tumor durch drei wichtige Mechanismen: Regulation der tumorassozierten Angiogenese, Aktivierung einer organismus- und tumorspezifischen Immunantwort und durch die direkte Stimmulation des Tumorwachstums als autokriner Faktor.
Angiogenese ist das Wachstum neuer oder bereits existierender Blutgefäße sowie von Mikrokapillaren. Normalerweise findet dieser Prozess während der

Embryonalentwicklung oder der Wundheilung statt; man findet dieses Phänomen aber auch in allen größeren Tumoren.
Chemokinen werden bei der Angiogenese entscheidende Bedeutung beigemessen, daher teilt man sie auch in pro- und antiangiogene Chemokine ein. In gesundem Gewebe herrscht ein Gleichgewicht zwischen pro- und antiangiogenen Chemokinen. Wird das Gleichgewicht durch einen äußeren Einfluss gestört, z.B. zugunsten der proangiogenen Chemokine, so wird ein Tumorwachstum begünstigt (Belperio J.A. et al, 2000; Moore B.B., et al, 1998).

7.5.1.3 Stickstoffmonoxid (NO)

NO ist ein weiteres wichtiges Signalmolekül, das in vielen verschiedenen Geweben eine Reihe physiologischer Prozesse reguliert. Dazu gehören Vasodilatation, neuronale Funktion, Entzündung und die Immunantwort. NO hat nur eine sehr kurze Halbwertzeit von einigen Sekunden. Es wird mit Hilfe eines Enzyms mit dem Namen NOS hergestellt. Dieses Enzym convertiert Arginin und O_2 in Citrullin und NO. NADPH ist ein notwendiger Co-Faktor.
NO vermittelt eine Vielzahl biologischer Effekte. Daher ist es auch verständlich, daß eine Dysregulation der NOS, die verantwortlich ist für eine Reihe von biologischen Prozessen, zu einer Vielzahl verschiedener Krankheiten führen kann (Kolb J.P., 2000).

7.5.2 Ziel der Untersuchungen

Es wurde die Expression verschiedener Parameter der Angiogenese in aktivierten DLD-1 Kolonkarzinom Zellen untersucht. Um diese Zellen zu aktivieren wurden zwei Stimuli verwendet.
Im ersten Teil der Studie wurden die Zellen Pyrrolidine-Dithiocarbamate (PDTC) ausgesetzt. PDTC ist ein AP-1 aktivierendes Reagenz, welches für die Behandlung von Darmkrebs in Kombination mit Zytostatika vorgeschlagen wurde (Chinery, R., 1997; Bach, S.P.2000; Della Ragione, F. 2000).
Im zweiten Teil wurden Effekte von NO, welches ein immunmodulierendes Radikal ist, untersucht. NO wird enogen im unmittelbaren Umfeld des Tumors produziert.

Die Expression der induzierbaren Stickoxidsynthase und die Produktion von Stickoxid (NO) korreliert mit der Angiogenese bei verschiedenen Krebserkrankungen (Melanome, Tumore im Hals und Kopfbereich und Darmkrebs) (Thomsen, L.L., et al. 1995; Cobbs, C.S.,et al.1995; Klotz, T., et al. 1998Liu, C.Y., et al. 1998; Thomsen, L.L.,et al. 1998; Gallo 0., et al. 1998).
Da tumorbegünstigende Funktionen von NO mit vermehrter Angiogenese in Verbindung gebracht werden (Jenkins, D.C., et al 1995; Ambs, S., et al. 1998), wurden die Effekte von NO hinsichtlich der Produktion von ausgesuchten Chemokinen, die an der Steuerung des Tumorwachstums beteiligt sind, untersucht.

7.5.3 Untersuchungen und Ergebnisse

Der Produktion von Interleukin-8 (IL-8), Häm Oxygenase-1 (HO-1), und dem vaskulären endothelialen Wachstumsfaktor (VEGF) wird zunehmend größere Bedeutung im Rahmen der Regulation der Immunantwort bei Entzündung, Infektion und Tumorwachstum zugemessen (Mitchell J.F., et al. 2001; Ferrara, N. et al. 1999; Doi, K., et al. 1999 ; Nishie, A., et al. 1999). Ziel dieser Arbeit war die Untersuchung der Regulation dieser Botenstoffe in *vitro* durch Verwendung der humanen Dickdarm Karzinom Zellinie DLD-1. Die Substanz Pyrrolidine Dithiocarbamate (PDTC) verstärkt nicht nur die durch Tumor Nekrose Faktor-α (TNF-α) vermittelte Ausschüttung von IL-8, sondern induziert auch als alleiniger Stimulus die IL-8 Sekretion. Mutationsanalysen des IL-8 Promotors und „Electrophoretic Mobility Shift“ Untersuchungen (EMSA) zeigten, daß die Aktivierung des Transkriptionsfaktors AP-1 (Aktivator Protein-1) und die Bindungsaktivität von konstitutiv aktiviertem Nuclear Faktor-κB (NF-κB) in DLD-1 Zellen für die PDTC induzierte IL-8 Expression zwingend erforderlich waren. Weiterhin war PDTC in der Lage in DLD-1 Zellen neben IL-8 auch die Expression von HO-1 und VEGF zu verstärken. Die Induktion von IL-8 durch PDTC war nicht nur auf DLD-1 Zellen beschränkt, sondern wurde auch in Caco-2 Zellen (ebenfalls Dickdarmkrebszellen) und in humanen mononukleären Blutzellen beobachtet. Die Verwendung von PDTC wird seit kurzem als Kozytostatikum für die Behandlung von verschiedenen bösartigen Tumoren, unter ihnen auch Darmkrebs, vorgeschlagen (Chinery, R., 1997; Bach, S.P.2000; Della Ragione, F. 2000). Aus unseren Versuchen läßt sich ableiten, daß die Induktion von IL-8, HO-1 und VEGF die therapeutische Anwendung dieser Substanz nachteilig

beeinflussen könnte. Dies ergibt sich daraus, daß die Faktoren IL-8 und VEGF durch ihre proangiogene Wirkung und die HO-1 durch ihre antiapoptotische Wirkung das Tumorwachstum fördern.

Die Expression der induzierbaren Stickoxidsynthase und die Produktion von Stickoxid (NO) korreliert mit der Angiogenese bei verschiedenen Krebserkrankungen darunter Melanome, Tumore im Hals und Kopfbereich und Darmkrebs (Thomsen, L.L., et al. 1995; Cobbs, C.S.,et al.1995; Klotz, T., et al. 1998; Liu, C.Y., et al. 1998; Thomsen, L.L.,et al. 1998; Gallo 0., et al. 1998). Da tumorbegünstigende Funktionen von NO mit vermehrter Angiogensese in Verbindung gebracht werden (Jadeski, L.C., and Lala, P.K. 1999; Kerbel, R.S. 2000), wurden die Effekte von NO hinsichtlich der Produktion von ausgesuchten Chemokinen, die an der Steuerung des Tumorwachstums beteiligt sind, untersucht. Zu diesen Chemokinen gehören das proangiogene IL-8 (Koch, A.E., et al. 1992) sowie das tumorsuppressive Interferon induzierbare Protein-10 (IP-10) (Luster, A.D., et al. 1995) und das Monokin induziert durch Interferon-γ (MIG) (Addison C.L., et al. 2000). Diese Chemokine werden, nach Stimulation mit IL- 1β und Interferon-γ (IFN-γ) von DLD-1 Zellen, ausgeschüttet. Unter diesen Bedingungen wird die IL-8 Freisetzung alleine durch IL-1β vermittelt nicht aber durch INFγ. Im Gegensatz zu IL-8 hängt die Sekretion von IP-10 und MIG von der Aktivierung durch IFNγ ab. Die Effekte von NO wurden analysiert indem DLD-1 Zellen mit dem NO-Donor DETA-NO inkubiert wurden. DETA-NO besitzt eine Halbwertzeit von 16,5h und simuliert damit die Effekte der NOS. Synthese und Freisetzung von IL-8 wurden durch die Behandlung mit NO stark gesteigert. Außerdem wurde in Zellen die dem NO-Donor ausgesetzt wurden die basale Sekretion des VEGF signifikant verstärkt. Dies steht im Gegensatz zur IL-1β/IFNγ-induzierten Produktion von IP-10 und MIG, beide wurden durch Koinkubation mit NO unterdrückt. Ebenso wurde die Expression der IFNγ abhängigen iNOS in DLD-1 Zellen von NO unterdrückt. Die vorliegenden Daten ergänzen vorherige Studien, in denen NO mit Tumorangiogenese und verstärkten Tumorwachstum in Verbindung gebracht wird. Die NO vermittelte Induktion von IL-8 und VEGF, ebenso wie die Verminderung der IP-10 and MIG Expression, könnte zu diesem Phänomen beitragen. Unsere Studien stützen die Hypothese, daß spezifische Inhibitoren der iNOS therapeutischen Nutzen bei humanen Neoplasien haben könnten.