Oligonucleotides suppress PKB/Akt and act as superinductors of apoptosis in human keratinocytes

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ABSTRACT

DNA oligonucleotides (ODN) applied to an organism are known to modulate the innate and adaptive immune system. Previous studies showed that a CpG-containing ODN (CpG-1-PTO) and interestingly, also a non-CpG-containing ODN (nCpG-5-PTO) suppress inflammatory markers in skin. In the present study it was investigated whether these molecules also influence cell apoptosis. Here we show that CpG-1-PTO, nCpG-5-PTO, and also natural DNA suppress the phosphorylation of PKB/Akt in a cell-type-specific manner. Interestingly, only epithelial cells of the skin (normal human keratinocytes, HaCaT and A-431) show a suppression of PKB/Akt. This suppressive effect depends from ODN lengths, sequence and backbone. Moreover, it was found that TGFX-induced levels of PKB/Akt and EGFR were suppressed by the ODN tested. We hypothesize that this suppression might facilitate programmed cell death. By testing this hypothesis we found an increase of apoptosis markers (caspase 3/7, 8, 9, cytosolic cytochrome c, histone associated DNA fragments, apoptotic bodies) when cells were treated with ODN in combination with low doses of staurosporin, a well-known pro-apoptotic stimulus. In summary the present data demonstrate DNA as a modulator of apoptosis which specifically targets skin epithelial cells.

INTRODUCTION

Redundancy seems to be a basic principle of living matter providing an economic handling with limited resources. An example of this is the desoxyribonucleic acid (DNA) that was for a long time only considered as carrier of genetic information. It is more than one hundred years ago since William Coley found that a preparation of heat inactivated streptococci induces some immunological response in cancer patients (1,2). It took until 1984 since bacterial DNA itself was identified to convey this effect (3). This observation demonstrated that information within the DNA molecule is not restricted to the genetic code. Later it was found that a specific sequence, a motif displaying an unmethylated cytidine-phosphate-guanosine (CpG) is able to activate the immune system (4). The fact that the CpG motif has a much lower incidence in human DNA than predicted by random base utilization and the increased amount of methylated cytidine residues makes this motif an ideal pathogen-associated molecular pattern (PAMP) recognized by the endosomal toll-like receptor-9 (5–8). Clinically, CpG and also some non-CpG oligonucleotides (ODN) are currently tested as immunomodulators for immune protection, allergic response, vaccination booster and in antitumor therapy (9). For these applications much efforts have been placed on the development of structurally modified ODN. Particularly, the substitution of one of the nonbridging oxygen atoms bound to phosphorus by sulfur is very commonly used. These phosphorothioates offer an increased serum stability and resistance against nucleases (10). Moreover, phosphorothiates can form mRNA–DNA duplexes which elicit RNase H activity triggering the degradation of mRNA (11). This mechanism is considered to play an important role in most antisense-based applications.

Both the presence of CpG-motifs and the modification of the DNA-backbone with phosphorothiates are described to stimulate pro-inflammatory responses (4,12–14). Therefore, it was a surprise that a CpG-ODN and a non-CpG-ODN both with phosphorothioate backbones suppressed pro-inflammatory markers in skin.

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keratinocytes and attenuated the cutaneous delayed-type hypersensitivity response in a mouse model (15). These findings speak for a tissue-specific mode of action. As the duration of inflammation is frequently regulated by apoptosis of the effector cells (16), we investigated the impact of CpG-ODN, and non-CpG-ODN with and without phosphorothioate backbones on apoptosis in a variety of epithelial and non-epithelial cells. Of note, it was found that epithelial cells of the skin namely, HaCaT cells, A-431 cells and normal human keratinocytes show PKB/Akt suppression and facilitated apoptosis in response to ODN. This effect was modulated by ODN backbone, sequence and length. These findings suggest that ODN can act as modulators of skin homeostasis.

MATERIALS AND METHODS

Reagents
ODN with phosphorothioate backbone were synthesized and purified by BioSpring GmbH (Frankfurt/Main, Germany), reconstituted in water and stored at −20°C. ODN were given to the cells at the indicated concentration without DNA complexing reagents. Hybridization of CpG-1-PTO and its reverse strand CpG-1-PTO-rev was performed as described (15). DNA from Escherichia coli, Clostridium perfringens and DNA from salmon sperm were purchased from ICN Pharmaceuticals (Heidelberg, Germany) and dissolved in water. Staurosporin (STS) was purchased from Sigma (Taufkirchen, Germany). TGFβ was purchased from PeproTech (Hamburg, Germany).

Cell culture
Spontaneously immortalized human keratinocyte cell line (HaCaT) (a generous gift by Prof. Fusenig, German Cancer Research Institute, Heidelberg, Germany) was cultured in carbonate buffered Hank’s medium with 5% fetal calf serum, 0.35 g/l glutamine (Invitrogen, Paisley, UK) and 1% penicillin–streptomycin solution (Biochrom KG, Berlin, Germany) at 37°C in 5% CO₂ atmosphere. The human epithelial carcinoma cell line A-431, the human epithelial kidney line HEK-293, the kidney fibroblast cell line Cos-7 (all purchased from the American Type Culture Collection, ATCC), the human sebocyte cell line SZ95 (kindly provided by Christos Zouboulis, Universitätsklinikum Benjamin Franklin, Berlin) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Karlruhe, Germany) containing 1% penicillin–streptomycin solution and 10% FCS. Normal human keratinocytes (NHK) and fibroblasts were isolated from skin specimen of cosmetic surgeries. NHK were propagated in serum-free keratinocyte medium (Gibco) and fibroblasts were held in RPMI 1640 medium (Biochrom, Berlin, Germany) with 10% FCS and 1% penicillin–streptomycin solution. Likewise, G-361 melanoma cells (ATCC), derived from a 31-year-old Caucasian male skin specimen of cosmetic surgeries. NHK were propagated in endothelial growth medium (EGM, Lonza, Wuppertal, Germany). The medium was renewed twice a week. All experiments were done in agreement with the local ethics commission.

Plasmids and transfection
The human dominant-negative TLR-9 plasmid was kindly provided by Hermann Wagner (Technische Universität München, München, Germany) (17). The dominant-negative flag-tagged MyD88 was kindly provided from Tularik (San Francisco, USA) (17). Empty pcDNA-3 served as control vector. Transient transfection was performed using A-431 cells plated in 6-well multidishes at a confluence of 70% using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturers’ instructions. The following day, cells were treated with 4 μM CpG-1-PTO or nCpG-5-PTO. After 30 min proteins were extracted as described.

Mechanical stimulation
Stretching of in vitro cultured cells was performed as described previously (18). Briefly, silicone elastomer MED-4011 (Armando Medizin Technik, Düsseldorf, Germany) was stirred, poured into preformed teflon matrices and allowed to polymerize. For allowing cell attachment silicone dishes were treated with 5.7% KOH in methanol for 5 min in order to neutralize the polymerization-derived HCl. After washing with double-distilled water, silicone dishes were coated with 2% arginine for 2 h to facilitate cell attachment (19) and afterwards rinsed with PBS. Subsequently, the dishes were incubated with fetal calf serum for 2 h. After withdrawal of the serum, cells were plated in flexible silicone chambers and incubated for 24 h under regular conditions. Prior to the application of mechanical stretch, the cells were held for 24 h under serum-free conditions. For the last hour 4 μM ODN were added to the cells. Then the silicone chambers were extended to 10% for 5 min. After stretch stimulation, protein samples were prepared at the indicated time intervals.

Immunoblotting
For detection of PKB/Akt and caspase 3 and 8 (antibodies from Cell Signaling Technology, Frankfurt, Germany), cells were lysed in 100 μl SDS sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromphenol blue], sonicated and boiled for 5 min, and separated on SDS–polyacrylamide gels. For detection of EGFR (Cell Signaling Technology), cells were scrapped into lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, 1 mM PMSF], sonicated and centrifuged. Protein concentration of the supernatant was determined (Biorad DC Protein Assay Kit, Biorad, München, Germany) and standardized using bovine serum albumin. Twenty micrograms of protein were mixed with SDS sample buffer and run on SDS–polyacrylamide gels. Consecutively, proteins were immunoblotted to a PVDF membrane. The membrane was blocked in blocking
buffer (TBS (pH 7.6), 0.1% Tween-20, 5% nonfat dry milk) for at least 3 h at 4°C followed by incubation with the primary antibody in TBS (pH 7.6), 0.05% Tween-20 and 5% BSA. The bound primary antibodies were detected using anti-mouse IgG-horseradish peroxidase conjugate and visualized with the ECL detection system (Amersham).

Cytosolic cytochrome c
In order to detect the amount of cytosolic cytochrome c, cells were fractionated as described (20). Briefly, 2 × 10^6 cells were trypsinized, washed and solubilized in 250 μl phosphate buffered saline (PBS). A mild lysis of the cell membrane was performed by adding 250 μl digitonin-solution (80 μg/ml sucrose solution, 500 mM). After vigorously vortexing for 10 s, cell extracts were centrifuged (1 min, 14,000 g, 4°C) and the protein content of the supernatant was determined as described under Immunoblotting. Thirty micrograms protein of the supernatant was applied to a commercial human cytochrome c immunoassay (R&D Systems, Wiesbaden, Germany).

Histone-associated DNA fragments
Apoptosis was quantified on the basis of cytoplasmic histone-associated DNA fragments using the Cell Death Detection ELISA (Roche, Mannheim, Germany) according to the manufacturer’s manual. In brief, cells were cultured in microwell plates (2 × 10^4 cells per 0.33 cm^2) and treated with increasing amounts of staurosporin (STS) in combination with different ODN. After 24 h cytosolic fraction (200 μg supernatant) was used as antigen source in a sandwich enzyme-linked immunosorbent assay with primary anti-histone antibody coated to a microtiter plate and secondary anti-DNA antibody coupled to peroxidase. Optical density was measured at 530 nm in an ELISA reader (MR 5000, Dynatech, Guernsey, UK).

Caspase 3/7 and 9 activity
Activity of caspase 3/7 and 9 was quantitatively assessed by using commercial assays (Caspase-Glo 3/7, Caspase-Glo 9, Promega, Mannheim, Germany). Cells were seeded in microplate wells and treated with staurosporin (STS) and ODN as aforementioned. After 24 h cells were lysed and luminogenic substrates specific for the different caspase species were added. Light emission was measured in a luminometer (Berthold, Bad Wildbad, Germany).

Statistical analysis
The grey levels of western blot bands were measured using ImageJ (Bethesda, USA). Data from western blots, histone associated DNA fragments, cytosolic cytochrome c and caspase 3/7 and 9 activity are given as means ± SD. Each column represents at least three independent experiments. Statistical analysis was performed using the Wilcoxon–Mann–Whitney U-test (BIAS, Frankfurt, Germany). Differences were considered significant at P < 0.05 indicated by asterisks. Figure legends indicate the data columns which were compared.

RESULTS
CpG-1-PTO and non-CpG-5-PTO suppress PKB/Akt
HaCaT keratinocytes were exposed to increasing amounts (0.5, 1, 2, 4 μM) of CpG-1-PTO or non-CpG-5-PTO for 30 min. Protein extracts were separated by SDS-PAGE and probed against phospho-PKB/Akt and total PKB/Akt for loading control, respectively. Figure 1A shows a dose-dependent suppression of basal PKB/Akt activation.

Fig. 1. Oligonucleotides suppress basal PKB/Akt activation. Protein extracts derived from HaCaT cells exposed to CpG-1-PTO and non-CpG-5-PTO were separated by SDS-PAGE. The blotted proteins were probed with anti-PKB/Akt-Ser473, anti-PKB/Akt-Thr308 and phospho-non-specific anti-PKB/Akt. (A) Concentration dependent effect of CpG-1-PTO and non-CpG-5-PTO. Cells were exposed to 0.5, 1, 2, 4 μM of CpG-1-PTO and non-CpG-5-PTO respectively for 30 min. (B) Statistical analysis of (A). Each column represents at least three independent experiments; standard deviations are indicated. Data were compared to untreated controls. * P < 0.05; solid bars, serine; white bars, threonine.
at both phosphorylation sites. Already a concentration of 0.5 μM nCpG-5-PTO led to a significant suppression of PKB/Akt (see statistical analysis in Figure 1B). In order to test the dynamic of this suppression HaCaT cells were exposed to 4 μM CpG-1-PTO (Figure 2A) and nCpG-5-PTO (Figure 2B), respectively for different times ranging from 5 min to 24 h. Statistical analysis in Figure 2C and D shows a rapid and robust downregulation by both oligos. Of note, the suppression of PKB/Akt phosphorylation lasted for at least 24 h. CpG-1-PTO and nCpG-5-PTO have a length of 20 nucleotides. In order to test if the lengths of the ODN molecules have an impact on PKB/Akt suppression different deletion mutants were tested (Table 1, Figure 3A–D). Deletion of flanking nucleotides from both ends of CpG-1-PTO reversed the PKB/Akt suppression in a length dependent manner. The hexamer CpG-6-PTO which still bears a complete CpG-motif shows no suppressive effect on PKB/Akt at 4 μM after incubation for 30 min. Vice versa nucleotide deletion of nCpG-5-PTO showed also a length dependent suppression of PKB/Akt (Figure 3B and D). ODN molecules with lengths from 20 down to 16 nucleotides showed a significant suppression of basal PKB/Akt phosphorylation.

Natural DNA suppress PKB/Akt

The aforementioned data show that both, CpG- and nCpG-ODN suppress basal PKB/Akt phosphorylation. In order to test if DNA in general conveys this effect,
we treated HaCaT keratinocytes with different natural occurring DNA derived from prokaryotes and eukaryotes. Figure 4A and B show that DNA from *Escherichia coli*, *Clostridium perfringens* and DNA derived from Salmon sperm suppress the basal phosphorylation of PKB/Akt. The observed effects are distinct but not as strong as for synthetic CpG-1-PTO. Furthermore, the natural DNA species offered no significant toxic effect on HaCaT cell in the tested concentrations as detected by the release of lactate dehydrogenase (LDH) (Figure S1, Supplementary Data). Figure 4C shows ethidiumbromide stained DNA samples separated by agarose gel electrophoresis. DNA derived from *Escherichia coli* gives a smear over the whole range and a slightly condensed section at approximately 10 kbp. This accumulation was more prominent for DNA derived from *Clostridium perfringens* and Salmon sperm. In order to test if shearing of DNA has an

Table 1. Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>CpG-1-PTO</td>
<td>5'-TCC ATG ACG TTC CTG ACG TT-3'</td>
</tr>
<tr>
<td>CpG-18-PTO</td>
<td>5'-TCC ATG ACG TTC CTG ACG-3'</td>
</tr>
<tr>
<td>CpG-16-PTO</td>
<td>5'-CC ATG ACG TTC CTG-3'</td>
</tr>
<tr>
<td>CpG-14-PTO</td>
<td>5'-CAT GAC GTT CCT-3'</td>
</tr>
<tr>
<td>CpG-12-PTO</td>
<td>5'-GAC GTT-3'</td>
</tr>
<tr>
<td>nCpG-5-PTO</td>
<td>5'-CCC CCC CCC CCC CCC-3'</td>
</tr>
<tr>
<td>nCpG-18-PTO</td>
<td>5'-CCC CCC CCC CCC CCC CCC-3'</td>
</tr>
<tr>
<td>nCpG-16-PTO</td>
<td>5'-CCC CCC CCC CCC CCC C-3'</td>
</tr>
<tr>
<td>nCpG-14-PTO</td>
<td>5'-CCC CCC CCC CCC CC-3'</td>
</tr>
<tr>
<td>nCpG-12-PTO</td>
<td>5'-CCC CCC CCC CCC-3'</td>
</tr>
<tr>
<td>nCpG-6-PTO</td>
<td>5'-CCC-3'</td>
</tr>
<tr>
<td>Scramb</td>
<td>5'-CTC TAG GAC TCT CTG GAC TT-3'</td>
</tr>
<tr>
<td>CpG-1-PTO-rev</td>
<td>5'-AAC GTC AGG AAC GTC ATG GA-3'</td>
</tr>
<tr>
<td>nCpG-5-PDE</td>
<td>5'-ccc ccc ccc ccc ccc ccc cc-3'</td>
</tr>
<tr>
<td>CpG-1-PDE</td>
<td>5'-tcc atg aag ttc atg aag tt-3'</td>
</tr>
</tbody>
</table>

Phosphorothioates (PTO) in capital letters, phosphodiesters (PDE) in small letters. CpG-motifs are underlined.
impact on the observed PKB/Akt suppression preparations of all three DNA species were treated with ultrasound. The effect of this treatment was monitored by agarose gel electrophoresis as aforementioned (Figure S2A, Supplementary Data). No changed effect on PKB/Akt suppression was detected suggesting that the molecular size is not a significant determinant in this case (Figure S2B, Supplementary Data).

PKB/Akt suppression depends from DNA sequence and backbone

In order to test what distinct information conveys the suppressive effect on PKB/Akt different synthetic 20-mer were tested (Figure 5A–C). It was found that both, the sequence and backbone are important for the observed effect. A CpG-1-molecule with phosphodiester bonds (CpG-1-PDE) offered no significant suppressive effect on PKB/Akt, whereas its phosphorothiotate counterpart shows the already noted strong inhibition. A scrambled sequence of CpG-1-PTO (Scramb) also shows strong inhibition. nCpG-5-PDE, which is a phosphodiester of nCpG-5-PTO, suppresses PKB/Akt in a moderate manner. Furthermore, the effect of a DNA hybrid was tested (Figure 5B). At first it was found that 4 µM of the reverse strand of CpG-1-PTO (CpG-1-PTO-rev) offered a slightly stronger suppressive effect than CpG-1-PTO indicating sequence specificity. This strong effect was maintained for the hybrid of CpG-1-PTO and CpG-1-PTO-rev, indicating that a single strand is sufficient to reduce PKB/Akt phosphorylation significantly.

Cell-specific suppression of PKB/Akt by CpG-1-PTO and nCpG-5-PTO

The aforementioned data were derived from experiments with HaCaT keratinocytes. In order to test if the suppression of PKB/Akt by CpG-1-PTO and nCpG-5-PTO is
cell specific also other cell species from different tissues were tested. Figure 6A shows the effect of CpG-1-PTO or nCpG-5-PTO on basal PKB/Akt phosphorylation on epithelial cells (A-431, HaCaT, NHK, HEK293, SZ95), fibroblasts (skin fibroblasts, Cos-7), melanoma cells (G361) and endothelial cells (HUVEC) after incubation for 60 min. The western blot analysis demonstrates a suppression of PKB/Akt phosphorylation in A-431, NHK and HaCaT cell as reference. Sebocytes (SZ95), fibroblasts derived from human skin, melanoma cells (G361), endothelial cells (HUVEC) and fibroblasts from kidney tissue (Cos-7) show no shift in PKB/Akt phosphorylation in response to ODN treatment. In order to test if classical CpG-effects are induced by CpG-1-PTO the murine macrophage cell line Raw264.7 was utilized (Figure 6B). It was found that treatment with 4 μM CpG-1-PTO leads to a time-dependent activation of PKB/Akt. This effect is well described leading to an anti-apoptotic response mediated via TLR-9, the CpG-receptor (21). A control experiment using nCpG-5-PTO devoid of a classical CpG-motif shows no activation of PKB/Akt in these cells. In sum, the results presented show a heterogenic response to CpG-1-PTO and nCpG-5-PTO depending on the cell species used. Of note, in epithelial cells derived from skin (HaCaT, NHK) and squamous epithelium (A-431) the ODN tested offer a clear suppression of basal PKB/Akt phosphorylation at both phosphorylation sites.

CpG-1-PTO and nCpG-5-PTO suppress induced levels of PKB/Akt and EGFR

The aforementioned data show that CpG-1-PTO and nCpG-5-PTO suppress the basal phosphorylation of PKB/Akt. Figure 7A shows a concentration dependent increase of PKB/Akt phosphorylation in response to TGFβ. This phosphorylation was attenuated by a preincubation with CpG-1-PTO or nCpG-5-PTO at a concentration of 4 μM for 30 min. As TGFβ induces
PKB/Akt phosphorylation via EGFR we investigated the effect of ODN on EGFR activation (Figure 7B). As expected, TGFα causes a dose dependent phosphorylation of EGFR. Interestingly, a pretreatment with CpG-1-PTO and particularly nCpG-5-PTO reversed this induction indicating a suppressive effect already at the surface receptor level. A statistical analysis performed for data given in Figure 7A and B shows significant reductions of PKB/Akt and EGFR in cells treated with 4 μM CpG-1-PTO or nCpG-5-PTO for 60 min. Then protein extracts were utilized for detection of PKB/Akt activation. (B) The murine macrophage cell line Raw267.7 known to activate PKB/Akt in response to CpG-DNA was treated with 4 μM CpG-1-PTO or nCpG-5-PTO for the indicated time intervals. Proteins were extracted and analyzed as aforementioned. The blots show representative results.

No impact of dominant-negative expression of TLR-9 and MyD88 on PKB/Akt suppression

CpG-DNA is recognized by a specific receptor, the toll-like receptor 9 (8) which mediates its signal via the adaptor protein MyD88 (22). In order to test if the here presented suppression of PKB/Akt by CpG-ODN and also non-CpG-ODN utilizes this signalling pathway A-431 cells were transfected with dominant-negative constructs for TLR-9 and MyD88 (Figure 8). Data given in Figure 8A and B show that mock transfected cells (pcDNA3) show a similar suppression of PKB/Akt as cells transfected with dominant-negative constructs for TLR-9 (ΔTLR-9) and MyD88 (ΔMyD88). These results demonstrate that the observed effects are rather independent from the TLR-9 signalling pathway.

CpG-1-PTO and nCpG-5-PTO suppress PKB/Akt activation in response to stretch

In the previous experiments ODN suppressed basal levels of PKB/Akt and after stimulation with a prototypical soluble mediator (TGFα). In the following a complete different stimulus, mechanical stretch, was applied to HaCaT cells (see ‘Materials and Methods’ section). Published data from our group already identified
mechanical stretch as strong inducer of PKB/Akt phosphorylation (23). Figure 9A shows that application of a stretching stimulus for 5 min induces PKB/Akt phosphorylation at both phosphorylation sites. A preincubation with 4μM CpG-1-PTO or nCpG-5-PTO for 30 min suppressed the stretch-induced phosphorylation. Of note, PKB/Akt induction by mechanical stretch shows some variation in the time kinetic resulting in a higher standard deviation (Figure 9B). Nevertheless, treatment with CpG-1-PTO or nCpG-5-PTO suppressed significantly the stretch-induced PKB/Akt levels at all tested time intervals.

CpG-1-PTO and nCpG-5-PTO sensitize for apoptosis

In the present study we have shown that treatment with CpG-1-PTO and nCpG-5-PTO suppresses basal and induced PKB/Akt—one of the key regulators in apoptosis. From this it could be construed that the ODN tested might change the sensitivity of cells against proapoptotic stimulation. This issue was addressed by using a combination of ODN and staurosporin—a well-known inducer of apoptosis. Data given in Figures 10 and 11 show that CpG-1-PTO and nCpG-5-PTO increase the pro-apoptotic effects staurosporin in HaCaT cells. In Figure 10A the application of staurosporin for 24h led to a significant increase of cleaved caspase 8 and 3 fragments in a dose dependent manner. When staurosporin and 8μM ODN were applied at the same time the appearance of cleaved caspases shifted to lower staurosporine concentrations indicating an accelerated onset of apoptosis. By using a quantitative detection kit for caspase 3/7 activity these results were confirmed: 8μM CpG-1-PTO and nCpG-5-PTO significantly amplified the effect of staurosporin (Figure 10B). These results were corroborated by measuring the cytosolic levels of cytochrome c (Figure 10C). A treatment with increasing concentrations of staurosporin alone for 14h led to a concentration dependent increase of cytosolic cytochrome c as measured by ELISA. In the presence of 8μM CpG-1-PTO the amount of cytosolic cytochrome c at 0.5μM staurosporin almost doubles. This effect was even more pronounced for 8μM nCpG-5-PTO: at 0.1 and 0.5μM staurosporin a significant increase of cytochrome c was measured. At 1.0μM staurosporin the measured levels of cytochrome c decreased again indicating advanced apoptosis. On the morphological level treatment with 1μM staurosporin for 8h induced the enhanced formation of apoptotic bodies as visualized by bisbenzimide staining (1μg/ml) (Figure 10D).
presence of 8 μM CpG-1-PTO this effect was considerably enhanced (Figure 10D).

The release of cytochrome c from mitochondria marks the onset of the intrinsic apoptosis triggering initiator caspase 9 activation. Quantitative detection of caspase 9 activity showed a significant amplification of the staurosponin effect (Figure 11A). These data are coherent in respect to results shown in Figure 10C documenting the activation of caspase 3/7 which are downstream from caspase 9. A late event in apoptosis is the degradation of DNA which can be quantitatively measure by the formation of histone associated DNA fragments. Figure 11B shows that CpG-1-PTO and nCpG-5-PTO enhance the pro-apoptotic effect of staurosporin while the hexamers of both ODN (CpG-6-PTO, nCpG-6-PTO) do not indicating that the apoptosis sensitizing effect of ODN depends on the molecule length. Finally, the effect of ODN on apoptosis was tested in the murine macrophage cell line RAW264.7 (Figure 11C). In contrast to skin epithelial cells CpG-1-PTO protect RAW264.7 cell from staurosporin induced apoptosis, an effect already documented (21).

**DISCUSSION**

In the present study we show that CpG-ODN, nCpG-ODN and also natural DNA suppress PKB/Akt

Fig. 8. No effect of ΔTLR-9 and ΔMyD88 on PKB/Akt suppression by CpG-1-PTO and nCpG-5-PTO. (A) A-431 cells were transfected with pcDNA3 and dominant-negative constructs of TLR-9 (ΔTLR-9) and MyD88 (ΔMyD88), respectively. After 24 h cells were treated with 4 μM CpG-1-PTO and nCpG-5-PTO for 30 min. Then protein extracts were utilized for detection of PKB/Akt activation. (B) represents the statistical analysis of (A). Each column represents at least three independent experiments; standard deviations are indicated. Data of each set were compared to transfection controls. *P < 0.05; solid bars, serine; white bars, threonine.
phosphorylation in epithelial skin cells. This is interesting as a recent study shows that a CpG-ODN from the same subclass (CpG-B) activates PKB/Akt in the mouse macrophage cell line RAW264.7 with an anti-apoptotic effect (21). This experiment was confirmed by us with using CpG-1-PTO. Likewise to this experiment others have found that CpG-ODN delay the onset of apoptosis in neutrophil granulocytes (24). Surprisingly, in our experimental setting using epithelial cells we found that both, CpG-ODN and also non-CpG-ODN sensitize cells for apoptosis. These results support current data showing that ODN trigger unexpected responses in epithelial skin cells and epithelial tissues (15). In this article it was demonstrated that the same ODN as used in the present study offer anti-inflammatory properties.

Besides the huge amount of data documenting the effect of CpG ODN on human cells there is increasing evidence that also some non-CpG ODN have physiological significance (9). Similarly, to CpG ODN also non-CpG ODN can exert an immune stimulatory effect in human leukocytes (25). Interestingly, the mode of stimulation differs between both ODN entities, and in contrast to CpG ODN non-CpG ODN support a Th2-biased response (26,27). Of note, the signaling of certain non-CpG-ODN require the presence of functional TLR-9 (27), which is surprising as previous work suggested that only CpG ODN, but not non-CpG ODN, trigger TLR9-mediated signaling (17,28,29). In contrast our data show that suppression of PKB/Akt in skin keratinocytes by CpG ODN, and non-CpG ODN seems to

![Graph](image)

**Fig. 9.** Stretch-induced PKB/Akt activation is suppressed by CpG-1-PTO and nCpG-5-PTO. (A) HaCaT cells were seeded in flexible silicon dishes, treated with 4 μM CpG-1-PTO or nCpG-5-PTO for 60 min and then mechanically stretched for 5 min as described under ‘Materials and Methods’ section. Proteins were extracted after 0, 10 and 30 min and analyzed for PKB/Akt activation. (B) represents the statistical analysis of (A). Each column represents at least three independent experiments; standard deviations are indicated. Data were compared to the referring controls. *P < 0.05; solid bars, serine; white bars, threonine.
be independent from TLR-9 signaling as displayed by dominant-negative expression of TLR-9 and the adaptor protein MyD88. Instead a dependency from ODN lengths, sequence and backbone was demonstrated. The relevance of these parameters was also shown for immunostimulation by non-CpG ODN (25). Similar to our findings phosphorothioates with lengths longer than 18 nucleotides offer high stimulatory effects on B-cells. Interestingly, these authors found thymidine-rich sequences more effective than sequences with high amounts of cytosine. Our data regarding PKB/Akt suppression show that a sequence solely consisting of cytosines (nCpG-5-PDE) is more effective than CpG-1-PDE with an amount of almost 30% thymidines. Although, ODN with phosphodiester bondings and also naturally occurring DNA derived from bacteria and salmon sperm have the potential to suppress PKB/Akt the presence of phosphorothiates amplified the efficacy.

The kinase PKB/Akt plays an important role in the suppression of apoptotic cell death (30,31). Proximal from surface receptors, the phosphoinositide 3-OH kinase (PI3K) conveys activation of PKB/Akt via phosphoinositide-dependent kinases (PDK). It has been demonstrated that PDK-1 phosphorylates PKB/Akt at threonine-308 (32), whereas the mechanism of the serine-473 phosphorylation is still under debate (33,34). Treatment with ODN as performed in this study causes suppression at both relevant phosphorylation sites,

Fig. 10. CpG-1-PTO and nCpG-5-PTO amplify staurosporin-induced caspase 3, 7, 8 activity, cytochrome c liberation and formation of apoptotic bodies. HaCaT cells were treated with 8 μM CpG-1-PTO or nCpG-5-PTO in the presence of 0.1, 0.5 and 1 μM staurosporin (STS). (A) After 24 h proteins were extracted and analyzed for degradation of caspase 3 and 8. The blot shows representative results. (B) After 24 h caspase 3/7 activity was assessed using a commercial assay as described under ‘Materials and Methods’ section. Activity of untreated cells was set to 100%. Each bar represents the mean of four parallel experiments; the standard deviations are indicated (* P < 0.05). (C) After 14 h a cytosolic extract was analyzed for cytochrome c. Each bar represents the mean of four parallel experiments; the standard deviations are indicated (* P < 0.05) (D) Cells were treated with 8 μM CpG-1-PTO and 1 μM STS for 8h. Then cells were fixed and apoptotic bodies were stained using bisbenzimide (1 μg/ml). Experiments were repeated with similar results.
Fig. 11. CpG-1-PTO and nCpG-5-PTO amplify staurosporin-induced caspase 9 activity and cytoplasmic histone-associated DNA fragments in HaCaT cells—CpG-1-PTO protects RAW264.7 cells from apoptosis. HaCaT cells were treated with 8 μM CpG-1-PTO or nCpG-5-PTO in the presence of 0.1, 0.5 and 1 μM staurosporin (STS). (A) After 24 h caspase 9 activity was assessed using a commercial assay as described under ‘Materials and Methods’ section. Each bar represents the mean of four parallel experiments; the standard deviations are indicated (*P < 0.05). (B) After 24 h cytoplasmic histone associated DNA fragments were assessed as described under ‘Materials and methods’ section. The effect of 20-mers (CpG-1-PTO, nCpG-5-PTO) was compared to hexamers (CpG-6-PTO, nCpG-6-PTO). Each bar represents the mean of four parallel experiments; the standard deviations are indicated (*P < 0.05). (C) The murine macrophage cell line Raw267.7 was treated with STS and different amounts of CpG-1-PTO. After 24 h cytoplasmic histone associated DNA fragments were assessed as aforementioned. Each bar represents the mean of four parallel experiments; the standard deviations are indicated (*P < 0.05).
although the lack of only one of these sites abrogates PKB/Akt signalling (35). PKB/Akt is known to control a plethora of molecules involved in apoptosis (31,36). Interestingly, treatment with ODN in the absence of an additional pro-apoptotic stimulus is not sufficient to trigger apoptosis. Moreover, in the presence of staurosporin the onset of apoptosis becomes facilitated. Our data provide evidence for an activation of the mitochondrial apoptosis machinery as documented by elevated cytochrome c levels in response to ODN treatment. Suppression of PKB/Akt leads to activation of the BCL-2 homology-3 domain-only proteins BIM and BAD which in turn mediate the release of cytochrome c (37,38). The release of cytochrome c from mitochondria marks the onset of the intrinsic apoptosis with subsequent activation of caspase-9 and caspase-3 (39). Furthermore, the activation of caspase-8, the increase of histone associated DNA fragments and the presence of apoptotic bodies indicate a massive induction of apoptosis at different stages.

More distal we found a suppression of the epidermal growth factor receptor (EGFR) by CpG-1-PTO, and particularly nCpG-5-PTO after stimulation with TGFz. The EGFR is characterized as a key element in the downstream signaling to PKB/Akt (40–42). Rockwell et al. (43) showed that the polyanionic nature of phosphorothioates enables interaction with proteins of the cell surface. Particularly, a specific inhibition of protein tyrosine receptors including EGFR was found. We speculate that the high amount of EGFR on epithelial cells may contribute to the selective action of ODN presented in this article. Our findings suggest that ODN and particularly phosphorothioates are interesting pharmacological compounds in the treatment of hyperproliferative skin diseases such as psoriasis or actinic keratosis. It could be speculated that ODN containing preparations help to lower the concentration of pro-apoptotic compounds with toxic side effects maintaining similar clinical effects.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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