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Sphingosine 1-phosphate is involved in cytoprotective actions of calcitriol in human fibroblasts and enhances the intracellular Bcl-2/Bax rheostat

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Calcitriol is originally known to decrease proliferation rates of several carcinoma cells, partly via induction of apoptosis. On the other hand, the secosteroid is revealed to protect some cell types like thyrocytes, HL-60 cells and melanocytes against programmed cell death. Here we report that calcitriol despite its strong antiproliferative effect on human dermal fibroblasts did not induce apoptosis in these cells. In contrast, calcitriol possessed an antiapoptotic action in dermal fibroblasts. Thus, the ability of the apoptotic stimuli TNFα/actinomycin and C_2-ceramides (C_2-Cer) to induce programmed cell death was drastically diminished in the presence of calcitriol. Moreover, we identified sphingosine 1-phosphate (S1P) as a downstream mediator of calcitriol for its cytoprotective property. Thus, the secosteroid could not protect fibroblasts from apoptosis in the presence of *N,N*-dimethylsphingosine (DMS), which inhibits sphingosine kinase, the crucial enzyme to form S1P. Like calcitriol, S1P in different concentrations did not induce fibroblast apoptosis and moreover drastically decreased the rates of apoptotic cells after treatment with TNFα/actinomycin. As S1P has been identified to modify the Bcl-2/Bax ratio in epithelial cells and keratinocytes, we also measured the expression of these proteins in dermal fibroblasts revealing an increased Bcl-2 level after stimulation with S1P while the Bax protein expression was not modified. In conclusion, calcitriol H was revealed to protect human fibroblasts from apoptosis by formation of S1P resulting in a changed Bcl-2/Bax ratio.

1. Introduction

Calcitriol is a promising therapeutic agent as it potently decreases proliferation and induces differentiation of several cell types. Even the treatment of carcinoma cell lines and xenografts like breast, colon and prostate carcinoma and several forms of melanoma results in a growth inhibition and tumor reduction (Diaz et al. 2000; James et al. 1996; Park et al. 2000; Welsh et al. 1998; Osborne and Hutchinson, 2002). Most of the effects of calcitriol are mediated by interaction with cytosolic vitamin D_3 receptors which then dimerize and translocate into the nucleus (Boyan et al. 2003). There, they bind to specific elements on calcitriol-responsive genes, regulate transcription and influence proliferation as well as life-and-death decisions. Consistently, in many cells and tumor cell lines it has been shown, that calcitriol leads to an induction of the cyclin kinase inhibitors p15, p16, p27 and p21 followed by a cell cycle arrest at the G_1-S transmission point (Osborne and Hutchinson, 2002). The differentiation-inducing effect of the secosteroid is mediated by an increased formation of c-fos while the levels of c-myc are diminished (Johansen et al. 2003). In prostate, breast and colon carcinoma cell lines, calcitriol moreover induces apoptosis by secretion of tumor necrosis factor α (TNFα) (Osborne and Hutchinson, 2002). TNFα binds to the p55 receptor followed by a recruitment of different associative proteins leading to a final activation of caspases and cell death (Wallach et al. 1999). Additionally, a further apoptotic signal transduction pathway of calcitriol/TNFα is initiated by activation of a neutral sphingomyelinase that hydrolyzes the membrane lipid sphingomyelin to ceramides (Dbaibo et al. 2001; Luberto et al. 2002). Ceramides show differentiation-inducing properties and have been identified as strong inductors of apoptosis by activation of caspases (Gomez-Munoz et al. 1994; Muller-Wieprecht et al. 2000). Besides these proapoptotic actions of calcitriol, there is also evidence of an opposed cytoprotective effect in HL-60 cells, thyrocytes, keratinocytes and even melanocytes (Kleuser et al. 1998; Wang and Studzinski, 1997; Xu et al. 1993, Sauer et al. 2003). Recently, it has been suggested that the antiapoptotic mechanism of calcitriol is a consequence of sphingosine 1-phosphate (S1P) formation. Thus, calcitriol enhances sphingosine kinase activity in HL-60 cells and keratinocytes creating the lipid mediator S1P by phosphorylation of sphingosine (Kleuser et al. 1998; Manggau et al. 2001). S1P is not only enhanced in response to calcitriol but also prevents keratinocytes and melanocytes from apoptosis induced by different stimuli (Manggau et al. 2001). It should be mentioned that S1P, besides its antiapoptotic action, is a potent and multiple cell and tissue mediator involved in growth, differentiation, migration and even organogenesis (Huwiler et al. 2000; Spiegel et al. 1998). Some effects, mainly apoptotic and cytoprotective

processes, are intracellularly performed, others by binding to a family of specific G protein coupled cell surface receptors (Takuwa et al. 2002; Ancellin et al. 2002).
In the context of cytoprotection it is noteworthy that members of the Bcl-2 family were identified as downstream mediators of calcitriol and S1P (Goetzl et al. 1999; Sauer et al. 2003; Wagner et al. 2003). These different proteins are fixed by hydrophobic chains in the mitochondrium membrane and show pro- as well as antiapoptotic properties. Thus, activated Bax releases cytochrome c from mitochondrial stores by the formation of channels or the opening of the permeability transition pore (Qian et al. 1997). Cytochrome c forms a complex with apaf-1 which associates procaspases for proteolytic activation. As Bcl-2 is capable to inhibit both mechanisms of cytochrome c release by Bax, an increase in the Bcl-2/Bax ratio counteracts apoptosis. On the contrary, in a variety of cells, where calcitriol induces apoptosis, a decrease of the Bcl-2/Bax ratio either via downregulation of Bcl-2 or via translocation of Bax to the mitochondrium has been reported (Diaz et al. 2000; James et al. 1996).

2. Investigations and results

2.1. Effects of calcitriol on growth, viability and apoptosis of human fibroblasts

Calcitriol has been indicated to inhibit the growth of a variety of cells. In accordance to other studies, our results clearly indicate a strong growth inhibition of human dermal fibroblasts induced by calcitriol (Fig. 1A). While a concentration of 10 nM did not influence proliferation rates, thymidine incorporation of proliferating fibroblasts was reduced by 50% and even 80% after exposure to 100 nM, respectively 1000 nM of calcitriol (Fig. 1A). Depending on the cell type the antiproliferative property of calcitriol is either connected with an induction of apoptosis or independently mediated. Despite an inhibition of cell growth, even an antiapoptotic action of calcitriol has been reported in keratinocytes and HL-60 cells. In fibroblasts the effect of calcitriol on apoptosis or cytoprotection has not been examined, therefore it was of great interest to further investigate underlying mechanisms. Measurement of cytotoxicity by MTT assays clearly confirmed that potent growth-inhibitory concentrations of calcitriol did not lead to a significant loss of cell viability (Fig. 1B). Furtheron, the influence of calcitriol on programmed fibroblast death was examined by flow cytometry. As characteristic of early apoptosis, we measured translocation of phosphatidylserine using FITC-conjugated annexin V. To discriminate between early as well as late apoptotic cells, cell staining by the nonvital dye propidium iodide (PI) was simultaneously measured. Our findings provide evidence that the rate of apoptotic cells is not enhanced by calcitriol in the physiologic concentration range up to 100 nM indicating that its growth inhibitory property is not due to fibroblast damage (Fig. 1C).

2.2. Protective property of calcitriol in dermal cells against apoptosis induced by TNFα and cell-permeable ceramides

Our results indicate that the antiproliferative action of calcitriol is not accompanied by an induction of apoptosis. To examine whether the secosteroid possesses a potential protective role in human dermal cells, we treated fibroblasts with calcitriol followed by apoptotic stimuli, namely TNFα and C_2-ceramides (C_2-Cer). As shown in Fig. 2A, a single treatment of fibroblasts with TNFα (20 ng/ml) did not enhance apoptosis. As TNFα besides its action as apoptosis inductor, in some cells is also capable to synthesize antiapoptotic proteins, an inhibitor of protein synthesis, actinomycin D, was added (Polunovsky et al. 1994). Indeed, a 16 h treatment with actinomycin D alone increased the number of apoptotic cells, which was drastically enhanced by the combination of TNFα (20 ng/ml) and actinomycin D (0.1 mg/ml) (Fig. 2A). The annexin V^+/PI^- section, indicating early apoptotic cells, was accelerated 5 times, the number of annexin V^+/PI^+ cells (late apoptosis) 3 times by an exposure to TNFα/actinomycin D (Table). As calcitriol mediates its antiapoptotic actions in HL-60 cells and keratinocytes via genomic mechanisms, a 24 h pretreat-

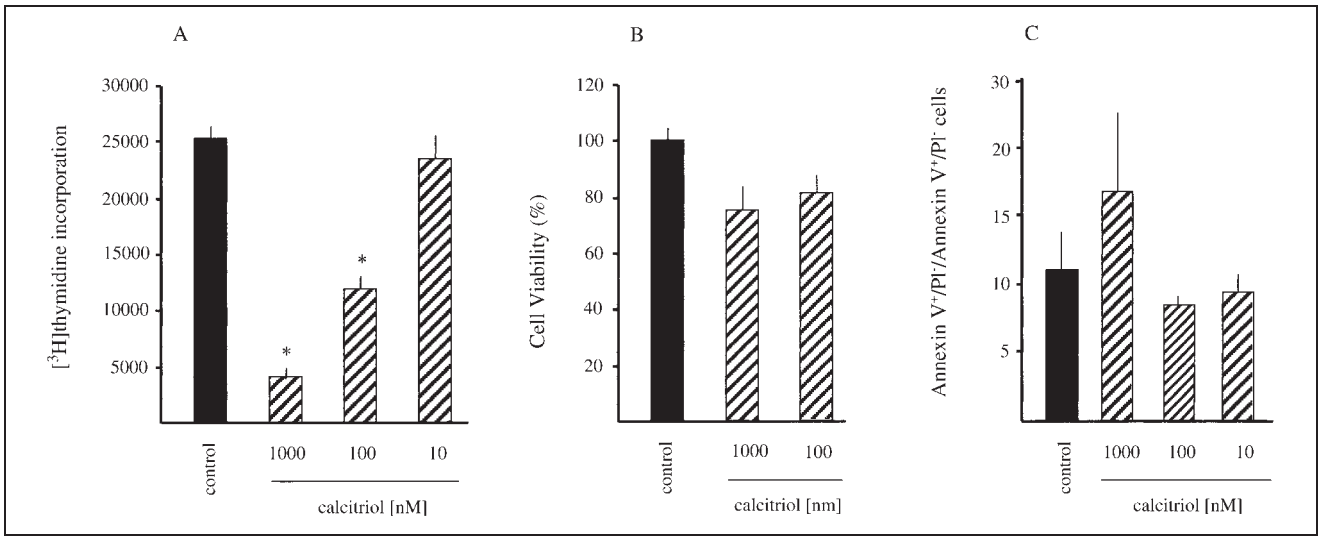

Fig. 1: Effect of calcitriol on proliferation, viability and apoptosis of human dermal fibroblasts. Human fibroblasts were treated with the indicated concentrations of calcitriol for 24 h and pulsed with [^{3}H]thymidine. Then, [^{3}H]thymidine incorporation into DNA was determined (A). After a 24 h-incubation of fibroblasts with calcitriol, viability was determined by a MTT-dye-exclusion assay as described in Experimental (B). Human fibroblasts were treated with calcitriol for 24 h. Then, double staining with Annexin V and PI was performed as described in Experimental (C)

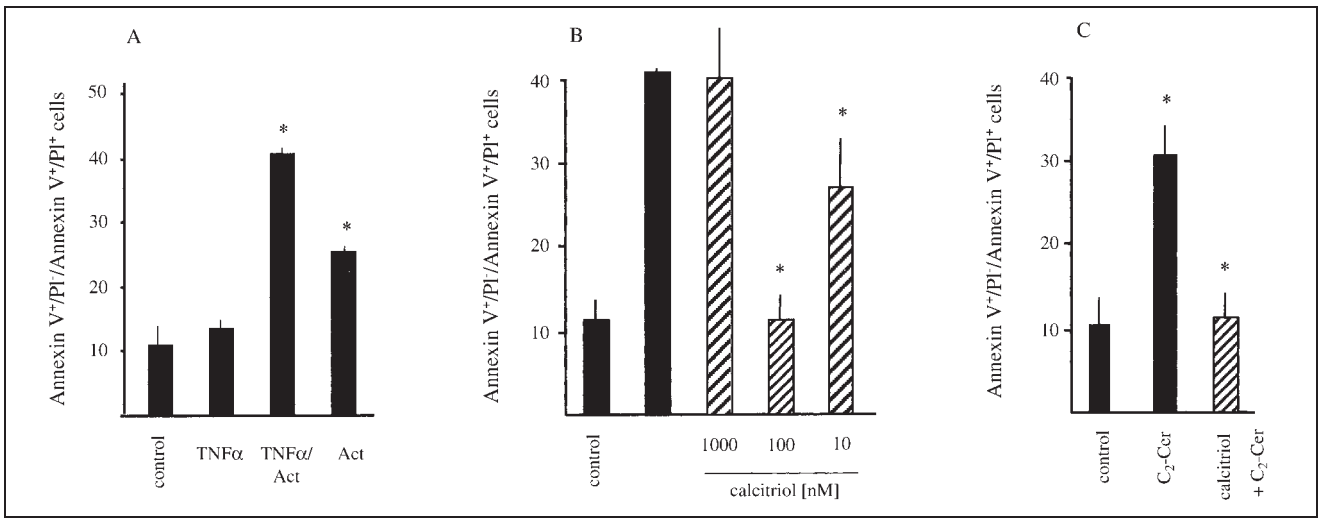

Fig. 2: Cytoprotective action of calcitriol on human dermal fibroblasts. Apoptosis of human dermal fibroblasts was induced by a 16 h treatment with TNFα (20 ng/ml), actinomycin D (Act, 100 μg/ml) or a combination of 20 ng/ml TNFα and 100 μg/ml Act (A). Human fibroblasts were treated with the indicated concentrations of calcitriol for 24 h. followed by an induction of apoptosis (B). Fibroblasts were treated for 24 h with the indicated concentrations of calcitriol. Then, apoptosis was induced by a 3 h treatment with C_2-Cer (50 μM), and double staining with Annexin V and PI was performed (C). Data are the means and standard deviations of triplicate assays. (* P 0.05)

ment interval with the secosteroid was chosen. As shown in Fig. 2B, 100 nM calcitriol fully protected fibroblasts against TNFα/actinomycin D-induced apoptosis. The numbers of both early as well as late apoptotic cells were reduced to control levels (Table). To further substantiate a protective role of calcitriol independently from the apoptotic stimulus, fibroblasts were incubated with C_2-Cer. Indeed, C_2-Cer (20 μM) led to an enhanced number of apoptotic cells, which was drastically diminished when cells were pretreated with calcitriol (Fig. 2C). These findings indicate for the first time a cytoprotection of fibroblasts by calcitriol independent of the apoptotic stimulus.

2.3. Role of S1P in the cytoprotective action of calcitriol in human fibroblasts

In HL-60 cells, the underlying mechanism by which calcitriol mediates its antiapoptotic property is a genomic activation of sphingosine kinase, phosphorylating ceramide degradation products to cytoprotective S1P. To identify the influence of this sphingolipid on fibroblast apoptosis, cells were exposed to S1P. Indeed, S1P in a concentration range from 0.1–10 μM did not enhance the number of apoptotic dermal cells. When cells were preincubated with S1P and subsequently treated with TNFα/actinomycin D again, a similar protection against TNFα-induced programmed cell-death was seen like this in response to calcitriol. S1P was most potent in a concentration of 10 μM (Fig. 3B). In contrast, a 0.1 μM S1P was only capable to reduce the section of Annexin V^+/PI^+ but not this of Annexin V^+/PI^- cells (Table).

Typical representative flow cytometric analysis are presented in Fig. 4 revealing the protective effect of 100 nM calcitriol (Fig. 4A) as well as 10 μM S1P (Fig. 4B) against TNFα/actinomycin D-induced apoptosis.

We next investigated the involvement of S1P in the cytoprotective effect of calcitriol. Therefore, we measured whether treatment of fibroblasts with calcitriol is accompanied by an increase of intracellular S1P concentrations. Indeed, mass levels of S1P were enhanced after stimulation with calcitriol. Thus, S1P contents raised by more than 30% in response to a 24 h stimulation with calcitriol from 435 pmol/10^8 cells to 570 pmol/10^8 cells (Fig. 5A). To reveal that S1P is involved in the antiapoptotic action of calcitriol, we proved the cytoprotective effect of the secosteroid in the presence of *N,N*-dimethylsphingosine (DMS). This compound has been identified as a specific inhibitor of sphingosine kinase which counteracts the formation of S1P. Indeed, in the presence of DMS, the antiapoptotic property of calcitriol was drastically diminished indicating that S1P is involved in its antiapoptotic property (Fig. 5B).

Table: Apoptotic rates of fibroblasts after stimulation with TNFα/actinomycin (Act) with or without preincubation with the indicated concentrations of calcitriol or S1P (* P 0.05)

Stimulation	Annexin V^+/PI^- cells (in % ± SD)	Annexin V^+/PI^+ cells (in % ± SD)
control	4.5 ± 0.5	6.5 ± 2.2
TNFα/Act	23.7 ± 2.7	17.1 ± 1.4
TNFα/Act + calcitriol 1000 nM	18.8 ± 1.4	19.7 ± 2.2
TNFα/Act + calcitriol 100 nM	6.9 ± 2.0*	6.7 ± 0.6*
TNFα/Act + calcitriol 10 nM	14.2 ± 0.7*	5.0 ± 1.8*
TNFα/Act + S1P 10 μM	7.5 ± 0.4*	9.4 ± 0.6*
TNFα/Act + S1P 1 μM	7.7 ± 0.4*	10.8 ± 0.1*
TNFα/Act + S1P 0.1 μM	28.7 ± 4.3	11.0 ± 0.5

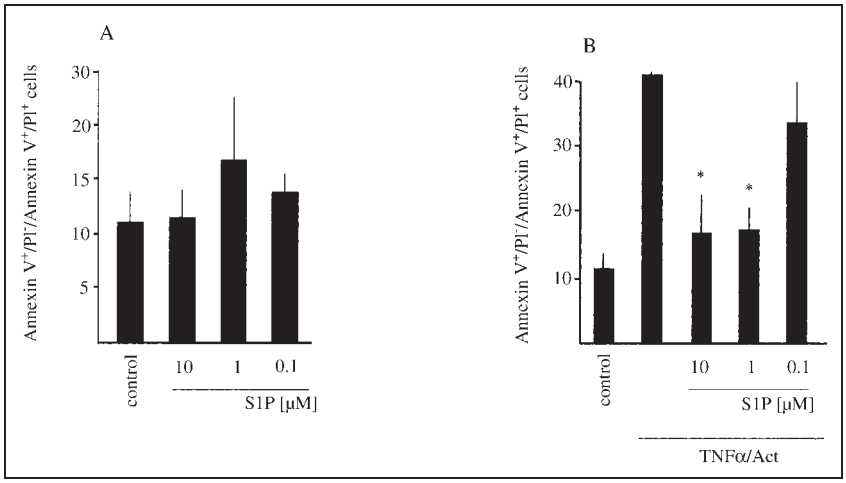

Fig. 3:
Effect of S1P on apoptosis and cytoprotection in human dermal fibroblasts. Human fibroblasts were treated with the indicated concentrations of S1P for 24 h. Then, double staining with Annexin V and PI was performed as described in Experimental (A). Cells were treated with the indicated concentrations of S1P for 24 h followed by an induction of apoptosis (B). Data are the means and standard deviations of triplicate assays. (* P 0.05)

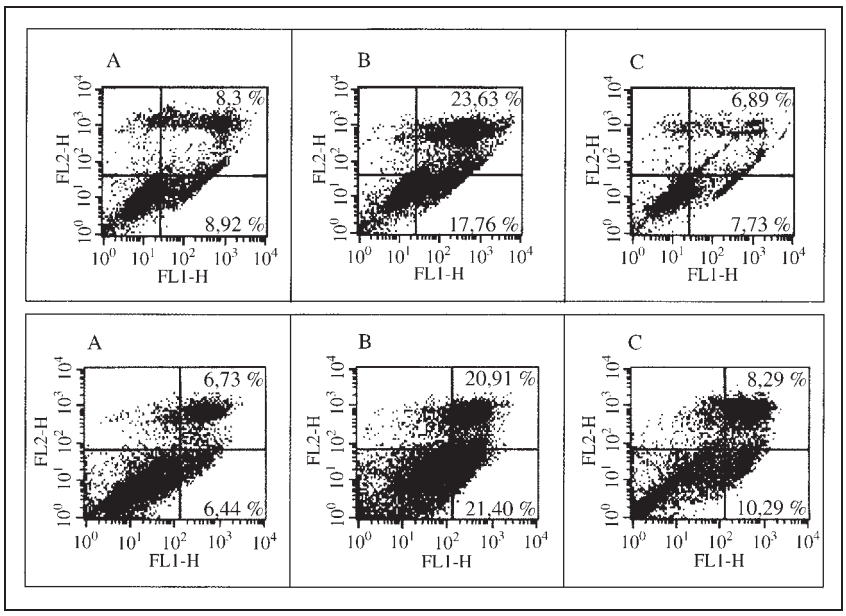

Fig. 4:
Flow cytometric analysis indicating a protective effect of calcitriol and S1P against TNFα induced apoptosis in human dermal fibroblasts. Cells were preincubated with either vehicle (A, B) or 100 nM calcitriol (C, upper panel) or 10 µM S1P (C, lower panel) for 24 h. Then apoptosis was induced by addition of TNFα (20 ng/ml) and actinomycin (100 µg/ml) for 16 h (B, C). Cells were harvested and double staining with Annexin V and PI was performed as described

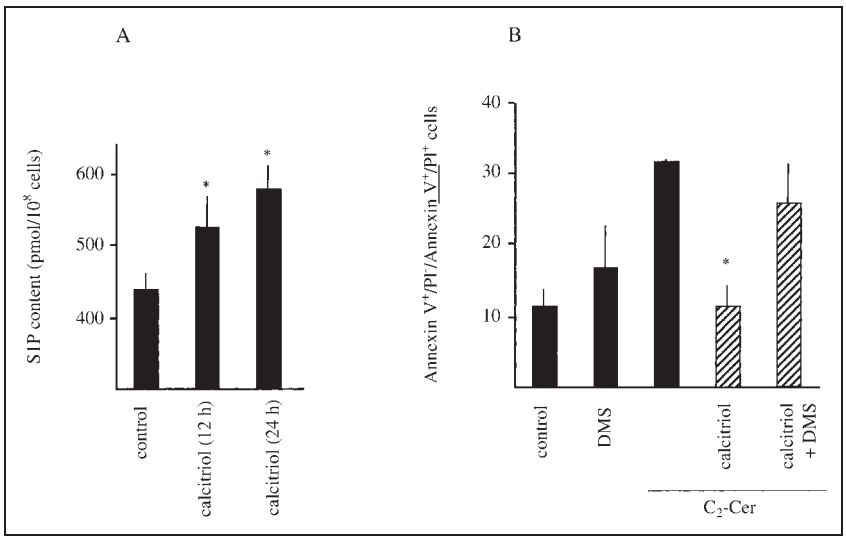

Fig. 5:
Effect of calcitriol on S1P formation (A) and involvement of S1P in the cytoprotection induced by calcitriol (B). Fibroblasts were treated with calcitriol (100 nM) for 12 or 24 h. Then S1P contents were measured as described (A). Fibroblasts were treated for 24 h with or without 5 µM of DMS and 100 nM of calcitriol. Apoptosis was induced for 3 h using C_2-Cer (50 µM). Then, double staining with Annexin V and PI was performed. Data are the means and standard deviations of triplicate assays. (* P 0.05)

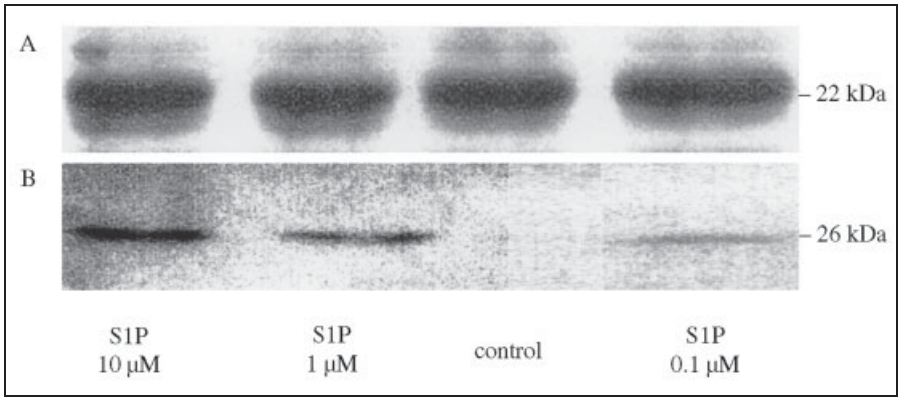

Fig. 6:
Influence of S1P on Bcl-2 and Bax protein expression. Total protein was obtained from human dermal fibroblasts after 40 h stimulation with S1P in the indicated concentrations. 10 μg protein per lane were loaded on a 12.5% polyacrylamide gel and western blot analysis was performed as described in Experimental. Individual bands represent protein expression of Bax protein at 22 kDa (A) and of Bcl-2 at 26 kDa (B)

2.4. Influence of S1P on the Bcl-2/Bax rheostat

A treatment of human keratinocytes with S1P could be associated with increased levels of Bcl-2 proteins, while expression of Bax proteins was not influenced (Manggau et al. 2001). Bcl-2 is known to prevent apoptosis by a reduced activity of the permeability transition pore and counteracts the effects of the proapoptotic Bax protein. As we revealed S1P as a potent cytoprotective agent in human dermal fibroblasts, we next measured the expression of both apoptosis control proteins in these cells. In western blot analysis of Bax protein, none of the tested S1P-concentrations caused a change of Bax-levels (Fig. 6A). In contrast, S1P influenced the expression of antiapoptotic Bcl-2. Thus, an increase of protein level at 26 kDa was visible after stimulation of cells with 10, 1 and 0.1 μM S1P, but did not occur in not-stimulated control cells. In summary, we identified calcitriol and S1P as cytoprotective agents in dermal fibroblasts. Calcitriol mediates this antiapoptotic property under involvement of S1P which modifies the Bcl-2/Bax ratio.

3. Discussion

Depending of the cell type, calcitriol possesses proliferative as well as growth inhibitory properties. For its antiproliferative effects, it is respected as a promising agent in the treatment of tumors (Diaz et al. 2000; James et al. 1996; Park et al. 2000; Welsh et al. 1998). Main mechanisms for the influence of cell growth are associated with changed life-and-death decisions by calcitriol. Thus, it shows a strong apoptotic effect in diverse melanoma and breast cancer cell lines as well as insulinoma cells (Okazaki et al. 1989). These studies revealed an influence of calcitriol on different signaling pathways involved in the regulation of apoptosis. Thus, calcitriol opposes the effect of the antiapoptotic acting insulin like growth factor (IGF) by down-regulating the IGF-receptor and enhancing the IGF-binding protein (Osborne and Hutchinson, 2002). Calcitriol has also been reported to activate the membrane sphingolipid degradating enzyme sphingomyelinase leading to an increase of intracellular ceramide-levels (Okazaki et al. 1989, 1990). Ceramides show strong apoptosis-processing properties as they release cytochrome c by an opening of the mitochondrial PT pore or activate the stress activated protein kinase SAPK (Jarvis et al. 1994). The way by which calcitriol leads to an enhancement of ceramide levels, could be at least in part, contributed to a secretion of TNFα, the well known inductor of apoptosis (Colell et al. 2002; Dbaibo et al. 2001; Luberto et al. 2002). Thus, downregulation of the endogenous sphingomyelinase activity as well as incubation with inhibitors of this enzyme prevented TNFα induced apoptosis (Luberto et al. 2002) and several studies indicate the secretion of TNFα in response to calcitriol (Geilen et al. 1997, 1996) as well as a cross talk between calcitriol and the TNFα pathway. Thus, pretreatment of MCF-7 cells with calcitriol potentiates the apoptotic action of TNFα (Rocker et al. 1994) and also leads to an increased expression of the TNFα-receptor, enhanced TNFα-induced nuclear factor ϰB-activation, and an increased release of lysosomal cathepsin B (Osborne and Hutchinson 2002).

But calcitriol is also capable to potently prevent cells like thyrocytes, HL-60 cells, keratinocytes and melanocytes from apoptosis (Kleuser et al. 1998; Manggau et al. 2001; Sauer et al. 2003, Wang et al. 1999; Xu et al. 1993). Here, we show that calcitriol, though it strongly sinks proliferation rates, also possesses mechanisms of maintaining cell viability of primary human fibroblasts. Interestingly, in these cells, calcitriol seems to form the sphingolipid mediator S1P from ceramide degradation products, which effects compensate apoptotic properties of ceramides. Thus, S1P protected fibroblasts from TNFα/actinomycin D induced apoptosis, and coincubation of calcitriol with DMS almost completely diminished the cytoprotection by this secosteroid against C_2-Cer induced apoptosis. It should be mentioned that DMS has been identified as a specific competitive inhibitor of sphingosine kinase and therefore is used to investigate the biologic role of S1P (Edsall et al. 1998).

As S1P is already characterized as a potent antiapoptotic agent in a variety of cells (Maceyka et al. 2002), it was not an unexpected finding that in HL-60 cells, keratinocytes and melanocytes, calcitriol mediates its cytoprotective effect by increasing the levels of intracellular S1P (Kleuser et al. 1998; Manggau et al. 2001; Sauer et al. 2003). A similar mechanism has been shown in endothelial cells which were stimulated with TNFα without additionally added actinomycin D (Xia et al. 1999). In conclusion with our findings in fibroblasts, endothelial cells were resistant to TNFα induced apoptosis as this cytokine not only induced sphingomyelin hydrolysis resulting in ceramide accumulation but also activated sphingosine kinase by genomic mechanisms.

This raises the question how S1P mediates its protective role. It is controversially discussed whether the cytoprotective role of S1P is a result of its binding to its G-protein coupled S1P-receptors (Takuwa et al. 2002) or if it acts as an intracellular mediator (Cuvillier et al. 1996). Although the present data show that calcitriol activates sphingosine kinase leading to an intracellular enhancement of S1P it cannot be excluded that it is transported into the extracellular

medium acting in an autocrine fashion via S1P-receptors. This mechanism was revealed by Ancellin and colleagues in vascular smooth muscle cells (Ancellin et al. 2002).

Moreover it is of great interest, that in fibroblasts, like in keratinocytes, calcitriol is capable of preventing apoptosis on both levels of origin, as not only TNFα but also ceramides were not able to induce programmed cell death in the presence of the secosteroid (Manggau et al. 2001). A variety of studies implicate that the ratio of cellular S1P and ceramide levels determines whether a cell undergoes apoptosis or not (Cuvillier et al. 1996, Kleuser et al. 1998). S1P prevents the activation of the apoptosis-processing stress-activated protein kinase (SAPK/JNK) pathway which is, at least in part, responsible for apoptotic cellular answers to ceramides (Cuvillier et al. 1996). In human and also murine fibroblasts, S1P is known to activate the extracellular signal regulated kinases Erk 1 and Erk 2 (Castillo et al. 2003, Cuvillier et al. 1996). Both kinases are members of the mitogen-activated protein kinase cascade which is also activated by several growth factors and associated with proliferative and cytoprotective cellular processes as they counteract SAPK activation (Castillo et al. 2003, Cuvillier et al. 1996; Pages et al. 2000).

Interestingly, we could identify another crucial intracellular antiapoptotic mechanism which is influenced by S1P. This is the well known mitochondrial system of the Bcl-2 family, in which there are both apoptosis-protective members (Bcl-2, Bcl-xl, Mcl-1) as well as proapoptotic proteins (Bax, Bcl-xs). Indeed, it has been clearly indicated, that in response to calcitriol and S1P the ratio of Bcl-2/Bax can be decreased or increased leading to apoptotic or antiapoptotic processes (Manggau et al. 2001; Sauer et al. 2003; Wagner et al. 2003; Wang et al. 1999). A decreased Bcl-2/Bax ratio results in an opening of the mitochondrial permeability transition pore (Antonsson et al. 1997). In the following there is a release of cytochrome c into the cytosol, which together with APAF1 results in the activation of caspases. But in contrast, calcitriol is capable to enhance Bcl-2 protein levels in thyrocytes as well as in keratinocytes, where it mediates an antiapoptotic action by stabilizing mitochondrial membranes (Manggau et al. 2001). Collectively, these findings present an antiapoptotic property of calcitriol and S1P in human fibroblasts under involvement of an increased Bcl-2/Bax ratio.

4. Experimental

4.1. Materials

Calcitriol was kindly donated by Dr. Lise Binderup (Leo-Pharmaceutical Products, Ballerup, Denmark). S1P was purchased from Calbiochem (Bad Soden, Germany), [*methyl*-^{3}H]Thymidine (35 Ci/mmol) from Amersham Pharmacia Biotech (Freiburg, Germany). DMS and C2-cer were received from Biomol Research Laboratory (Plymouth Meeting, PA). Annexin V-fluoresceine isothiocyanate (annexin V-FITC) and PI were obtained from Alexis (Grünberg, Germany). Actinomycin D, amphotericin B, aprotinin, BSA, dimethyl sulfoxide, dithiothreitol, Na_2EDTA, ethylenglycol-bis-(ß-aminoethylether)-*N,N,N,N*-tetraacetic acid, FBS, gentamicin sulfate, L-glutamine, ß-glycerolphosphate, HEPES, leupeptin, MTT, phenylmethylsulfonylfluoride, sodium orthovanadate, sodium pyrophosphate, trichloroacetic acid, Tris, Triton X-100 and Dulbecco's modified Eagles medium (DMEM) were purchased from Sigma (St. Louis, MO). TNFα was from Seromed Biochrom (Berlin, Germany). Mouse monoclonal anti-Bcl-2- and anti-Bax-antibodies and LumiGlo Reagent and peroxide were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG-antibody was from New England Biolabs (Beverly, MA).

4.2. Cell culture

To isolate human fibroblasts, juvenile foreskin from surgery was incubated at 37 °C for 2.5 h in a solution of 0.25% trypsin and 0.2% EDTA. Trypsinization was terminated by the addition of DMEM containing 10% FBS. Cells were washed with phosphate-buffered saline (PBS) and centrifuged at 250 × g for 5 min. The pellet was resuspended in DMEM by the addition of 10% FBS, 2 mM L-glutamine, 50 ng amphotericin B per ml and 20 µg gentamicin sulfate per ml. Fibroblasts were pooled from several donors and cultured at 37 °C in 5% CO_2. Only cells of the second to fourth passage were used for the experiments.

4.3. DNA-Synthesis

Fibroblasts (4×10^4 cells/well) were grown in 24-well plates for 24 h. Then medium was replaced by fresh DMEM/10% and cells were incubated with the indicated concentrations calcitriol for 24 h and pulsed with 1 µCi of [*methyl*-^{3}H]thymidine per well. After 23 h medium was removed and cells were washed twice each with PBS and ice-cold trichloroacetic acid (5%). The precipitated material was dissolved in 0.3 N NaOH-solution and incorporated [*methyl*-^{3}H]thymidine was determined in a scintillation counter (MicroBeta Plus, Wallac Oy, Turku, Finland).

4.4. MTT dye-reduction assay

Fibroblasts (8×10^4 cells per well), seeded into 24-well plates for 24 h, were incubated with test substances for 24 h at 37 °C in 5% CO_2. After the addition of 100 µl MTT solution (5 mg per ml) per well, the plates were incubated for another 4 h. The supernatants were removed and the formazan crystals were solubilized in 1 ml of dimethyl sulfoxide. The optical density was determined at 540 nm using a scanning microplate spectrophotometer (Multiscan Plus, Labsystems, Helsinki, Finland).

4.5. Annexin V binding and PI dye exclusion by flow cytometry

Fibroblasts (8×10^4 per well) were cultured in fibroblast basal medium (DMEM with 2 mM L-glutamin, 50 ng/ml amphotericin B and 20 µg/ml gentamicin sulfate). After incubation with the indicated agents cells were trypsinized and washed twice with binding buffer (10 mM HEPES/NaOH ph 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Apoptosis was determined by flow cytometric detection of phosphatidylserine translocation using fluoresceinlabeled annexin V (Vermes et al. 1995). To discriminate between early apoptotic cells (Annexin V+/PI−) as well as late apoptotic and necrotic cells (annexin V+/PI+), dye exclusion of the nonvital dye PI was simultaneously measured. Therefore cells were resuspended in binding buffer followed by the addition of annexin V-FITC (final concentration 0.5 µg/ml). The mixture was incubated for 10 min in the dark at room temperature, washed, and resuspended in binding buffer. Then PI was added (1 µg/ml) and samples were analyzed by bivariate flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg).

4.6. Mass measurement of S1P

S1P levels were determined as described (Ruwisch et al. 2001). Briefly, after stimulation with calcitriol (100 nM, 12 h), fibroblasts (2×10^6) were washed with PBS and scraped into 1 ml methanol containing 2.5 µl concentrated HCl. As internal standard dihydro-S1P (50 pmol) was added and lipids were extracted by addition of 1 ml chloroform and 200 µl 4 M NaCl. For alkalisation, 100 µl 3 N NaOH were added. The alkaline aqueous phase was transferred into a siliconised glass tube, and the organic phase was reextracted with 0.5 ml methanol, 0.5 ml 1 M NaCl and 50 µl 3 N NaOH. The aqueous phases were combined, acidified with 100 µl concentrated HCl and extracted twice with 1.5 ml chloroform. The organic phases were evaporated, and the dried lipids dissolved in 275 µl methanol/0.07 M K_2HPO_4 (9 : 1). A derivatisation mixture of 10 mg *o*-phthaldialdehyde, 200 µl ethanol, 10 µl 2-mercaptoethanol and 10 ml 3% boric acid was prepared and adjusted to pH 10.5 with KOH. 25 µl of the derivatisation mixture were added to the resolved lipids for 15 min at room temperature. The derivatives were analysed by a Merck Hitachi LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) using a RP 18 Kromasil column (Chromatographie Service, Langerwehe Germany). Separation was performed with a gradient of methanol and 0.07 M K_2HPO_4. Resulting profiles were evaluated using the Merck system manager software. The recovery of S1P was calculated using dihydro-S1P as standard.

4.7. Immunoblot analysis for Bcl-2/Bax

The expression of Bcl-2 and Bax was determined by western blot analysis using mouse monoclonal antibodies to human Bcl-2 or Bax. Fibroblasts (1×10^6) were cultivated in 10 cm dishes, scraped from the plates and suspended in PBS. The cells were collected by centrifugation and the resulting pellets were suspended in ice-cold lysis-buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM ethylenglycol-bis-(ß-aminoethylether)-*N,N,N,N*-tetraacetic acid, 1% Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerolphosphate, 1 mM sodium orthovanadate and 1 µg leupeptin per ml]. After incubation on ice for 30 min, samples were centrifuged at 13000 × g for 20 min. The Triton-soluble fraction was collected, and 15 µg of protein were subjected to a 12.5% polyacrylamide gel and transferred to nitrocellulose membranes. The blots were blocked in Tris-buffered saline/Tween 20 (1%) with 5% nonfat dry milk for 1 h at

37 °C, incubated with the primary antibodies for 3 h and a horseradish peroxidase-conjugated second antibody for 1 h at room temperature. Immune complexes were detected with an enhanced chemoluminescence detection method.

4.8. Statistical analysis

Data are the mean from triplicate assays and are expressed as mean ±SD. All experiments were repeated at least three times independently. Statistics were performed using Student's t test with $p < 0.05$ considered significant.

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