3. Discussion

Phospholipase D has been implicated in cellular proliferation but also differentiation and apoptosis. In hyperproliferative diseases the regulation of these processes is altered. *Psoriasis vulgaris* is one such hyperproliferative disease of the skin. Therefore, the mechanisms of regulation of phospholipase D in HaCaT keratinocytes as a model for basal keratinocytes was investigated. Tyrosine phosphorylation stimulates phospholipase D1 activity, but the enzyme itself is not phosphorylated in HaCaT keratinocytes. Furthermore, phospholipase D1 as well as several markers of differentiation are downregulated in ceramide-induced apoptosis in HaCaT keratinocytes. A second hyperproliferative disease, malignant melanoma shows enhanced expression of phospholipase D1 and phospholipase D2. Pamidronate, a member of the bisphosphonate class of therapeutics inhibits proliferation and induces apoptosis in melanoma cells.

3.1 Effect of tyrosine phosphorylation on phospholipase D1

Epidermal growth factor and other growth factors can induce phospholipase D activity. The epidermal growth factor receptor is a receptor tyrosine kinase and phospholipase D may therefore be activated by mechanisms involving tyrosine phosphorylation. Additionally, tyrosine kinase inhibitors abolish the induction of phospholipase D activity by hydrogen peroxide in neutrophils. The protein tyrosine phosphatase inhibitor sodium pervanadate was used to provoke an accumulation of tyrosine phosphorylated proteins. Tyrosine phosphorylation was assessed by Western blot using the monoclonal anti-phosphotyrosine antibody PY-20. Serum-induced HaCaT keratinocytes show an increase in tyrosine phosphorylated proteins upon treatment with 100 μM sodium pervanadate for 10 min. Preincubation for 5 min with 1 mM genistein, a broad range tyrosine kinase inhibitor completely abolishes tyrosine phosphorylation. The protein kinase C inhibitor staurosporine has no effect at a concentration of 100 nM. Sodium pervanadate is generated using sodium vanadate and hydrogen peroxide where superfluous hydrogen peroxide is destroyed using catalase. However, the use of this inhibitor in HL60 cells is controversial since hydrogen peroxide itself induces tyrosine phosphorylation in these cells. In contrast, in HaCaT keratinocytes no increase of tyrosine phosphorylation is observed by treatment with hydrogen peroxide at equal conditions as with sodium pervanadate. This shows specific accumulation of tyrosine phosphorylated proteins in HaCaT keratinocytes using sodium pervanadate. Sodium pervanadate treatment results in increased

phospholipase D activity in HL60 cells (BOURGOIN and GRINSTEIN, 1992). The activity of phospholipase D1 can be determined *in vitro* exploiting the transphosphatidylation activity of the enzyme (YANG *et al.*, 1967). Phosphatidylcholine-specific phospholipase D prefers primary alcohols in suspense of water to hydrolyse or alcoholyse the phosphatidyl-enzyme intermediate, generating the respective non-physiologic phosphatidylalcohol. Specificity for phospholipase D1 is achieved using an assay as described by BROWN *et al.* (1993). Defined substrate vesicles composed of phosphatidylethanolamine, phosphatidylinositol-4,5-bis-phosphate and phosphatidylcholine at a ratio of 18:1.4:1 (w/w/w) are incubated with cell lysates at a protein concentration of 0.4 µg/µl. The non-hydrolysable analogue of GTP, guanosine-5'-*O*-(3-thiotriphosphate) (GTPγS) at a concentration of 100 µM is used to activate monomeric G-proteins. Here, I can show that phospholipase D1 activity is significantly increased in lysates of sodium pervanadate treated HaCaT keratinocytes as well as in Ras transfected HaCaT keratinocytes or HL60 cells. Pretreatment with 1 mM genistein completely blocks this increase in all three cell lines tested. Sodium vanadate at 100 µM or 1 mM genistein added directly to the assay have no effect on phospholipase D activity *in vitro*. These results suggest a tyrosine phosphorylation dependent activation of phospholipase D1 in these cells. Overexpression of Ras has no effect on phospholipase D1 stimulation although tyrosine phosphorylation in Ras transfected HaCaT keratinocytes was strongly enhanced in comparison to untransfected cells (data not shown). Therefore, the Ras/Raf/mitogen activated protein kinase pathway is not involved in tyrosine phosphorylation dependent activation of phospholipase D1 in HaCaT keratinocytes.

Direct tyrosine phosphorylation of phospholipase D1 was shown in sodium pervanadate treated HL60 cells using immunoprecipitation (MARCIL *et al.*, 1997). Likewise, lysates of HaCaT keratinocytes, Ras transfected HaCaT keratinocytes and HL60 cells were immunoprecipitation using the specific anti-phospholipase D1 antibody αNChPLD1 (MÜLLER-WIEPRECHT *et al.*, 1998). Western blot of the precipitates with anti-phosphotyrosine antibodies shows a band with a molecular weight of $M_r = 110 \cdot 10^3$ for sodium pervanadate treated HL60 cells but not in 1 mM genistein pretreated HL60 cells. In HaCaT keratinocytes and Ras transfected HaCaT keratinocytes, however, no tyrosine phosphorylated band could be detected. Reprobing the blot with αNChPLD1 antibodies showed a strong band with a molecular weight of $M_r = 110 \cdot 10^3$ for all precipitates. Thus, phospholipase D1 is directly tyrosine phosphorylated in HL60 cells but not in HaCaT keratinocytes or Ras transfected HaCaT keratinocytes.

Tyrosine phosphorylated proteins accumulate in HaCaT keratinocytes upon treatment with the protein tyrosine phosphatase inhibitor sodium pervanadate. This accumulation is accompanied by increased phospholipase D1 activity *in vitro*. However, phospholipase D1 is not directly tyrosine phosphorylated in HaCaT keratinocytes in contrast to HL60 cells. The effects of treatment with sodium pervanadate can be abolished using the protein tyrosine kinase inhibitor genistein. Therefore, a tyrosine phosphorylated protein upstream of phospholipase D1 induces enhanced enzyme activity in response to sodium pervanadate treatment. As shown by Ras overexpressing HaCaT keratinocytes, the Ras/Raf/mitogen activated protein kinase pathway is not involved. In HEK-293 cells, phosphatidylinositol-4,5-bisphosphate levels are increased in response to sodium pervanadate treatment but this effect is evident only after longer time periods and higher concentrations than used in this study (RÜMENAPP *et al.*, 1998). Most probably, enhanced membrane translocation of RhoA is involved in increased phospholipase D1 activity (HOULE *et al.*, 1999). Although phospholipase D1 is directly phosphorylated in HL60 cells this pathway may act also in these cells since the activation of phospholipase D1 is of the same magnitude as in HaCaT keratinocytes.

3.2 Expression of phospholipase D in ceramide-induced apoptosis

Short-chain analogues of ceramide and several agonists indirectly elevating endogenous ceramide levels like the ceramidase inhibitor D-*e*-MAPP are potent inducers of apoptosis in keratinocytes (BEKTAS *et al.*, 1998; RAISOVA *et al.*, 2001b). Ceramides modulate phospholipase D1 activity via inhibiting protein kinase C or by reducing membrane translocation of ADP-ribosylation factor and Rho proteins (VENABLE *et al.*, 1996; ABOUSALHAM *et al.*, 1997). Moreover, the mRNA of phospholipase D1 is downregulated in C6 glioma cells after treatment with ceramide (YOSHIMURA *et al.*, 1997). Ceramide induced apoptosis in keratinocytes shows features of differentiation such as cornified envelope formation (WAKITA *et al.*, 1994). In this work, I demonstrate that phospholipase D1 and differentiation-associated genes are downregulated in ceramide-induced apoptosis in HaCaT keratinocytes.

The mRNA expression of several genes is assessed using RT-PCR of total RNA from HaCaT keratinocytes treated for 2 h, 6 h and 24 h with 30 µM *N*-acetyl-D-sphingosine (C_2-ceramide) or ethanol as vehicle control. Semi-quantitative analysis is achieved by scanning the reaction products stained with ethidium bromide using video densitometry and normalising with densities of respective expression of *ribosomal protein S9* mRNA. The

median of three independent experiments of treatment with C_2-ceramide was then set relative to the respective treatment with ethanol.

The transcription factor AP-1 is involved in apoptosis and differentiation (SAWAI *et al.*, 1995; ECKERT and WELTER, 1996). AP-1 is a dimer of proteins of the jun and fos protooncogene families. The jun family comprises c-jun, junB and junD whereas the fos family comprises c-fos, fosB, fra-1 and fra-2. I investigated the expression of the most prominent members c-jun and c-fos. The mRNA of *c-jun* is upregulated 4-fold after 6 h and 6-fold after 24 h of C_2-ceramide treatment. However, *c-fos* shows a transient upregulation after 6 h but normal levels after 24 h. The fos family member fosB shows twofold elevated mRNA levels after 6 h and 3.5-fold elevated levels after 24 h of treatment. These data suggest that AP-1 in ceramide-induced apoptosis is composed of c-jun homodimers and/or c-jun/fosB heterodimers whereas c-fos is not involved. Tissue transglutaminase is activated during apoptosis forming a cornified envelope. Its mRNA levels are not significantly altered by treatment with C_2-ceramide which is consistent with the observation that the expression of several apoptosis-involved proteins like caspases is not changed during the apoptotic process. Rather, these proteins are constant constituents of the cell. In contrast, transglutaminase 1 is a keratinocyte specific enzyme forming the cornified envelope in terminal differentiation with involucrin and loricrin being components of this cornified envelope. The upregulation of the expression of these three proteins as well as of several keratins such as keratin V is typical for terminal differentiation of keratinocytes. Furthermore, the promotors of these genes contain binding sites for AP-1. However, here I show that all four keratinocyte differentiation-associated genes are downregulated after 24 h of treatment with C_2-ceramide. Similarly, the transcripts of *phospholipase D1a* and *phospholipase D1b* are both downregulated after 24 h of treatment with C_2-ceramide in agreement with YOSHIMURA *et al.* (1997). The mRNA levels of phospholipase D2 are unchanged at all time points investigated.

AP-1 formed of c-jun/c-jun or c-jun/fosB may therefore act as inhibitor of expression of differentiation-associated genes or a repressor might specifically interact with these AP-1 complexes to abolish expression. The fact that both isoforms of phospholipase D1 are downregulated in a similar manner suggests the same regulatory mechanism. Differentiation and apoptosis are therefore two unrelated processes and similarities are rather occasionally. Especially the generation of the cornified envelope that occurs in both processes is performed by different transglutaminases using diverse substrates.

3.3 Regulation of phospholipase D in melanoma cells and melanocytes

Cancer is characterised by enhanced proliferation. Statistically, it has been suggested that a cell has to accumulate seven protooncogenic mutations before it is degenerated into a tumour cell (ALBERTS *et al.*, 1989). There are two main types of protooncogenes, one class consists of proteins involved in mitogenic signalling and cell cycle regulation and the second comprises proteins engaged in apoptosis. Both result in enhanced clonal proliferation, the first through enhanced cell division, the latter by decreased cell death. Escape from the innate immunoresponse can be achieved by this means if proliferation is higher than the number of cells removed by macrophages. Further mutations fostered by the already acquired mutations are necessary to change the tumour into metastatic growth. Several studies reported elevated phospholipase D activity or overexpression of phospholipase D isoforms in tumour cells. In this study, I demonstrate that melanoma cell lines exhibit elevated levels of phospholipase D1 activity and expression compared to primary cultured melanocytes.

The mRNA expression of phospholipase D isoforms was examined using RT-PCR. Expression of *phospholipase D1a, D1b* and *D2* is present in melanoma cell lines as well as in primary cultured melanocytes. However, comparing the levels in respect to *ribosomal protein S9* expression reveals higher expression of *phospholipase D2* in melanoma cell lines. Ectopic overexpression of phospholipase D2 was shown to provoke cytoskeletal reorganisation (COLLEY *et al.*, 1997). Slight differences in the expression levels of the *phospholipase D1a* and *D1b* splicing isoforms were observed. Whereas Mel2A cells and primary human melanocytes express both isoforms in equal amounts, A375, M186 and M221 express more *phospholipase D1b* than *phospholipase D1a*. Yet, there is no known difference in regulatory properties or subcellular localisation between both isoforms and therefore the rationale of this divergence remains obscure. The mechanism how phospholipase D overexpression is achieved in tumour cells is unknown. Elevated levels of phospholipase D1 are also observed using Western blot. Additionally, the protein expression of proteins directly regulating phospholipase D was investigated. The protein expression of protein kinase Cα was at comparable levels in all cells tested. RhoA expression is strong in melanocytes in comparison to melanoma cells whereas no ADP-ribosylation factor was found in melanocytes in contrast to melanoma cells. The latter is most probably due to low expression which is below the detection level of the antibody used although loading higher amounts of protein onto the gel did not result in detection of a band corresponding to ADP-ribosylation factor. For investigation of the subcellular

localisation of phospholipase D1, A375 cells were immunohistochemically stained using αNChPLD1 antibodies and the alkaline phosphatase anti alkaline phosphatase (APAAP) technique. The dyeing was cytosolic with no staining of the nucleus. Furthermore, the staining was strong around the nucleus suggesting a predominant association with the endoplasmic reticulum and Golgi apparatus.

Activation through regulatory proteins was determined *in vitro* using defined substrate vesicles and ethanol as mentioned before. The phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) was used to stimulate protein kinase C and the non-hydrolysable GTP analogue guanosine-5'-*O*-(3-thiotriphosphate) (GTPγS) was used to activate monomeric G-proteins. Phospholipase D activity is stimulated in a concentration dependent manner by TPA in the absence of GTPγS around two- to threefold at a concentration of 100 nM TPA in melanoma cells, whereas in melanocytes no significant activation is observed. Using increasing concentrations of GTPγS in the absence of TPA also showed a concentration dependent stimulation of phospholipase D activity. Maximal activation was about sevenfold at 50 μM GTPγS in melanoma cells and fourfold in melanocytes. Half-maximal stimulation was observed at about 20 nM TPA and 12 μM GTPγS. Melanocytes show low absolute phospholipase D1 activity in comparison to melanoma cells underscoring the results of lower phospholipase D1 protein expression. Combining both stimuli at 100 nM TPA and 50 μM GTPγS yielded phospholipase D activities around 19-fold for the melanoma cells but only fourfold for melanocytes. Depleting the cells from protein kinase C by long-term phorbol ester treatment resulted in a complete loss of the TPA response of phospholipase D activity. Moreover, no synergism was seen in combination with GTPγS. This demonstrates that TPA-mediated activation occurs directly by protein kinase C and is not upstream of monomeric G-proteins. Furthermore, TPA and GTPγS stimulated activity was strongly inhibited by micromolar concentrations of sodium oleate.

Increased phospholipase D1 activity in melanoma cells is attained through enhanced protein expression. In addition, although protein kinase Cα expression is not altered, activation of phospholipase D1 by phorbol ester is marginal in melanocytes in contrast to melanoma cells suggesting loss of an inhibitory factor of this interaction. However, it has to be mentioned that the growth media used in this study for melanoma cells and melanocytes differ substantially. The media are incompatible, therefore postconfluently grown cells were used to minimise media effects. Therefore, results of this work have to be recognised with caution and should be reevaluated using *in situ* mRNA hybridisation and immunohistochemistry of pigment cell *naevi* versus malignant melanoma.

3.4 Action of pamidronate on melanoma cells

Bisphosphonates are a class of therapeutics used to inhibit bone metastasis of mammary carcinoma. Initially, they were thought to act via inhibiting osteoclast proliferation and induction of apoptosis most probably by chelating calcium. Recently, mammary carcinoma cells themselves were shown to be affected by bisphosphonates (SENARATNE *et al.*, 2000). Nitrogen containing bisphosphonates were shown to inhibit the farnesyl diphosphate synthase probably by mimicking the diphosphate moiety (VAN BEEK *et al.*, 1999). They are therefore inhibitors of the synthesis of higher isoprenoids like geranylgeranyl diphosphate. The prenylation of monomeric G-proteins of the Ras superfamily like Rho proteins was shown to be reduced by bisphosphonate treatment. Geranylgeranylation of these proteins is required for their proper membrane association. Rho family proteins are engaged in cytoskeletal reorganisation and enhanced expression of several isoforms was observed in metastatic tumour cells (FRITZ *et al.*, 1999). Phospholipase D1 is regulated by Rho proteins and phosphatidic acid, the product of phospholipase D action can affect Rho proteins (CROSS *et al.*, 1996). Therefore, the inhibition of Rho proteins might provide a possibility to reduce metastasis through interference with this pathway. In this study, I demonstrate that melanoma cell proliferation can be reduced and apoptosis induced by pamidronate, a nitrogen-containing bisphosphonate.

Proliferation was measured assessing the number of adherent cells by crystal violet staining. Treatment with 100 µM pamidronate for 48 h resulted in significantly decreased cell numbers in A375, M186 and M221, but not in Mel2A. Cell numbers are reduced by 17 %, 11 % and 24 % in A375, M186 and M221, respectively. However, higher concentrations of 250 µM pamidronate did not further reduce cell numbers. Besides decreased proliferation, cytotoxicity can provoke reduced cell numbers. Therefore, the release of lactate dehydrogenase into the cell culture supernatant that is released upon membrane disruption as a typical feature of cytotoxicity is measured. No significant increase of lactate dehydrogenase activity in cell culture supernatants is observed following treatment with pamidronate for the cell lines tested. Nevertheless, a tendency of enhanced cytotoxicity is observable. Apoptosis is a controlled form of cell death. One of its features is the degradation of nuclear DNA resulting in histone-bound DNA fragments released into the cytosol of the cell. These complexes can be quantified in an ELISA using anti-DNA antibodies and anti-histone antibodies in combination. Treatment with pamidronate for 24 h resulted in significantly increased apoptosis in A375, M186 and M221 but not in Mel2A. Apoptosis is significantly elevated at 50 µM

pamidronate. At this concentration A375 show a 4.3-fold increase of DNA fragmentation compared to vehicle treated cells, M186 and M221 exhibit a 1.3-fold increase. Pamidronate at a concentration of 100 µM further augmented apoptosis with A375 evincing a 10-fold, M186 a 1.5-fold and M221 a 1.6-fold increase. The two cell lines M186 and M221 show low induction of apoptosis that might be due to the high basal apoptotic rate indicated by high amounts of histone-bound DNA fragments already in untreated samples. By using these values for normalisation this information is lost. Higher concentrations of 250 µM pamidronate did not further enhance DNA fragmentation in any of the cell lines tested. Another feature of apoptotic cell death is the activation of specific proteases, the caspases. Caspases reside inside cells as zymogens which are activated by proteolysis. Cleavage of procaspase-3 of $M_r = 32 \cdot 10^3$ generating fragments of $M_r = 20 \cdot 10^3$ and $M_r = 17 \cdot 10^3$ was shown using Western blot. A375, M186 and M221 but not Mel2A melanoma cells exhibit cleaved forms of caspase-3 after 24 h of treatment with 100 µM pamidronate. The induction of apoptosis by pamidronate can be diminished using farnesol or geranylgeraniol. Whereas farnesol reduces DNA fragmentation by 50 %, geranylgeraniol is more potent with decreasing DNA fragmentation to 75 % of pamidronate treated cells. This is in accordance with lack of geranylgeranylation and to a lesser extend farnesylation of proteins being responsible for the effect of pamidronate.

Mel2A and M221 melanoma cells were shown to be resistant to CD95 ligand- and ceramide-induced apoptosis (RAISOVA *et al.*, 2000). This resistance is achieved by shifting the ratio of the proapoptotic protein bax and the antiapoptotic protein bcl-2 towards bcl-2 and by this means inhibiting mitochondria-mediated apoptosis. Whereas Mel2A are resistant also to pamidronate, M221 are sensitive. To further investigate the role of the mitochondrial pathway in pamidronate-induced apoptosis, bcl-2 overexpressing A375 cells were used. The overexpression of bcl-2 in A375 was shown to convert their CD95 ligand-sensitiv phenotype into a resistant one (RAISOVA *et al.*, 2001a). However, pamidronate-induced DNA fragmentation was not altered in bcl-2 overexpressing A375 demonstrating that apoptosis provoked by pamidronate is independent of the mitochondrial pathway.

Several studies suggested that overexpression of Rho proteins is associated with tumour development and metastasis. The expression of Rho proteins was investigated using RT-PCR. RhoA is expressed at comparable levels in melanoma cells and melanocytes whereas RhoB is heterogeneously expressed. However, levels of RhoC mRNA are elevated in melanoma cells compared to melanocytes with Mel2A showing the highest expression. This suggests RhoC to be involved not only in metastasis but also in tumourigenicity.

The membrane association of Rho proteins is weakened when they are not geranylgeranylated. Prenylation of several proteins including the Ras superfamily was shown to be inhibited using nitrogen-containing bisphosphonates. I therefore investigated the distribution of RhoA as a representative of the Rho family proteins in cytosolic and membrane fractions of untreated and treated cells. Whereas RhoA levels were considerably elevated in the cytosolic fractions of all cell lines tested after 24 h and 48 h of treatment with 100 μM pamidronate, the membrane fractions showed inconsistent results. In A375 and Mel2A, membrane-bound RhoA was decreased after 48 h but not 24 h of treatment. Conversely, M221 showed reduced RhoA levels in membrane fractions after 24 h but not after 48 h of treatment. No change was observed in M186 melanoma cells. Membrane recruitment of Rho proteins is achieved not only through geranylgeranylation but also by protein-protein interactions. Increased protein expression of Rho proteins during pamidronate treatment may therefore be sufficient to counteract its effect and normalise levels of membrane bound Rho proteins.

These data demonstrate that pamidronate has only a weak impact on melanoma proliferation. In contrast, pamidronate treatment strongly induced apoptosis suggesting that it does not act as an unspecific cytotoxic agent. Moreover, one of the cell lines tested, Mel2A, is resistant against pamidronate mediated inhibition of proliferation and induction of apoptosis. This cell line is also resistant against CD95 ligand and ceramide induced apoptosis substantiated by low bax expression (RAISOVA *et al.*, 2001a). However, M221 cells are also resistant in this respect but are sensitive to pamidronate. Cytosolic RhoA levels are increased in all four melanoma cell lines after pamidronate treatment. Nevertheless, levels of membrane-bound RhoA are not or only slightly altered, suggesting that decreased geranylgeranylation is counteracted by increased expression of Rho proteins. Therefore, pamidronate might serve as a potent drug to prevent melanoma metastasis but some melanomas exhibit resistance and this resistance is not due to low bax expression. However, the more important question of modulation of the metastatic potential of these cell lines was not assayed in this study. The action of nitrogen-containing bisphosphonates on Rho proteins implies that metastasis may be suppressed independently of induction of apoptosis.