Expression of ectopeptidases in scleroderma

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Abstract

Objectives—To examine the expression and concentrations of three ectopeptidases likely to be involved in regulating the functional levels of adhesion molecules and the turnover of connective tissue components, in patients with scleroderma (systemic sclerosis) (SSc) and in normal individuals.

Methods—Monoclonal antibodies against these antigens were used for immunoperoxidase staining of cryostat skin sections and for flow cytometric (fluorescence activated cell sorter) analysis of cultured dermal fibroblasts grown from SSc patients and normal controls.

Results—Although neutral endopeptidase-24.11 (NEP) (CD10) was not detected in either SSc or normal skin, aminopeptidase N (APN) (CD13) and dipeptidyl peptidase IV (DPPIV) (CD26) were both readily visualised. However, DPPIV appeared to be present in smaller concentrations in the SSc biopsy specimens. Moreover, while fibroblasts grown in vitro from both SSc and normal skin also had similar concentrations of APN, the expression of DPPIV in the cultured SSc cells was found to be very much less than that present in the normal fibroblasts. It is noteworthy that NEP, which was not detected in the tissue sections, was nevertheless readily detected in fibroblasts in culture.

Conclusions—These results show that a number of cell surface proteases are expressed by dermal fibroblasts both in vivo and in vitro, and it is suggested that the marked downregulation of DPPIV in SSc could be at least partly responsible for the increased concentrations of adhesion molecules and matrix proteins associated with the molecular pathology of this disease.

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Systemic sclerosis (scleroderma) (SSc) is an autoimmune disease which involves endothelial cell damage and fibroblast activation, leading to the overproduction of extracellular matrix (ECM) and the development of fibrotic lesions.^{1–3} Examination of SSc skin biopsies, particularly at early stages of the disease process, have identified an infiltration and accumulation of leucocytes^{4 5} and a concomitant increase in cytokine concentrations,^{6 7} features which are well known to modulate fibroblast proliferation and matrix

synthesis.^{2 8–10} Other studies have shown that the pathogenesis of SSc is also associated with increased expression of adhesion molecules such as the intercellular adhesion molecule-1 (ICAM-1),^{11 12} which is a major ligand for the binding of leucocytes via the counter-receptors lymphocyte function associated antigen-1 (LFA-1) and Mac-1.^{13 14}

Ectopeptidases are widely distributed plasma membrane antigens the catalytic domains of which are enzymatically active at the extracellular surface. Neutral endopeptidase-24.11 (EC 3.4.24.11) (NEP) and aminopeptidase N (EC 3.4.11.2) (APN) are integral membrane proteins with zinc metallopeptidase activity. NEP cleaves peptide bonds amino-terminal to hydrophobic amino acids,¹⁵ and is found in high concentration in the brain, kidney, epithelia of lung and intestines, fibroblasts, neutrophils, and immature lymphocytes.¹⁶ cDNA and protein sequence analyses have shown that NEP is identical to CD10, the common acute lymphoblastic leukaemia antigen (CALLA).¹⁷ APN removes N-terminal peptides from nearly all unsubstituted oligopeptides, and has been found to be the same as the haemopoietic marker, CD13.¹⁸ In contrast, dipeptidyl peptidase IV (EC 3.4.15.1) (DPPIV) is a serine enzyme which liberates X-proline dipeptides from the amino terminus of peptides.¹⁵ The peptidase is identical to the leucocyte differentiation antigen CD26, a marker for a T cell subset,¹⁹ and may have a role in fibronectin mediated interactions of cells with components of the ECM,²⁰ and in neurohormonal peptide inactivation.²¹

The precise functions of these enzymes are not yet fully delineated, but it is possible that the modulation of their activities could play an important part in the molecular pathogenesis of SSc. For example, downregulation of protease expression could be partly responsible for the increased expression of ICAM-1 and increased leucocyte interactions reported in SSc.¹¹ In the present study we have therefore compared NEP, APN and DPPIV expression and distribution in normal skin and cultured dermal fibroblasts with that in SSc.

Patients and methods

PATIENTS

Seven female patients with SSc (aged 18–30 years; average 27) and seven normal individuals (four males, three females, aged 22–52 years; average 37) were studied. All the SSc patients had diffuse disease, characterised by early widespread proximal skin involvement and rapidly developing visceral abnormalities.

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All patients fulfilled the American College of Rheumatology preliminary criteria for SSc.²²

CELL CULTURE

Biopsies were taken from the affected skin of the SSc patients and matched areas from the controls. The biopsies were cut into 1–2 mm³ pieces and cultured in Dulbecco's modification of Eagles Minimal Essential Medium (DMEM), supplemented with L-glutamine 2 mmol/l, penicillin 100 U/ml, streptomycin 100 µg/ml and 10% fetal calf serum (FCS) in a humidified incubator at 37°C in an atmosphere of 5% carbon dioxide in air. The relatively low numbers of cells obtained in these initial outgrowths of the cultured biopsy tissue precluded analysis by flow cytometry, as described below. Therefore, in order to obtain a sufficiently large number of cells, when the cultures grew to confluence they were subcultured at a 1:6 dilution, by trypsinisation (0.25% trypsin in phosphate-buffered saline (PBS)), and recultured as described above. All fibroblast cultures were subsequently grown to confluence and used between passages 3 and 12.

IMMUNOCYTOCHEMICAL STAINING FOR LIGHT MICROSCOPY

Sections 6 µm thick were cut on a cryostat, mounted on glass slides coated with poly-L-lysine and air dried overnight. Slides were stored at –80°C. Before staining, the sections were fixed in chloroform:acetone (1:1) at –20°C for five minutes, washed with water and stained using an indirect immunoperoxidase method. Endogenous peroxidase was blocked with 0.1% phenylhydrazine hydrochloride for five minutes at 20°C. After washing with PBS, the sections were incubated with mouse anti-human monoclonal antibodies against NEP, APN and DPPIV (Serotec) for 60 minutes. The sections were incubated for a further 60 minutes with goat anti-mouse antibody conjugated to peroxidase (Dako Ltd). The sections were washed and developed for five minutes using 3,3'-diaminobenzidine tetrahydrochloride and then counterstained with Mayer's haematoxylin blue in lithium carbonate, cleared with HistoClear and mounted in DPX (BDH).

IMMUNOSTAINING OF FIBROBLASTS FOR FLOW CYTOMETRY ANALYSIS

In order to be able to assess quantitatively any possible differences in the levels of ectopeptidase expression, fibroblasts were grown from biopsy specimens and analysed by fluorescence activated cell sorting (FACS). The cells were grown to confluence in 75 cm² tissue culture flasks and were detached from the monolayers using EDTA 20 mmol/l in washing buffer (2% FCS in PBS) for 10 minutes at 37°C and scraping with a rubber policeman. They were centrifuged at 400 g for 10 minutes and the pellet resuspended by repeated pipetting to produce a single cell

suspension. The cells were fixed in freshly prepared 2% paraformaldehyde in PBS for 30 minutes at 20°C then washed, centrifuged, and resuspended in 10 ml of washing buffer and stored at 4°C. Approximately 10⁵ fixed cells were used for measuring the concentration of each antigen by FACS analysis. The cells were washed in buffer containing 2% FCS in PBS, centrifuged as described above and monoclonal antibodies against NEP, APN, and DPPIV were added for 60 minutes at 20°C. Secondary anti-mouse antibody conjugated to fluorescein isothiocyanate was applied for 30 minutes at 20°C. The fluorescence of 10 000 cells was measured using a Becton-Dickinson FACScan, in which identical settings and alignment were used in all experiments.

Results

IMMUNOLocalISATION OF ECTOPEPTIDASES IN NORMAL AND SSc SKIN

Immunoperoxidase staining of cryostat sections of control and SSc skin showed little reactivity for NEP (data not shown). In contrast, APN staining was readily apparent in both normal and SSc skin (fig 1A and B, respectively), in which it was localised in the papillary dermis, including fibroblast like cells in the papilla and the superficial and deep plexus of blood vessels. In the SSc skin, the localisation of APN was more prominent in the upper dermis (fig 1B).

Unlike APN, however, DPPIV showed marked differential staining between normal and SSc skin. Thus, while normal skin had extensive DPPIV immunoreactivity in numerous fibroblast like cells throughout the different layers (fig 2A), the intensity of this protease in SSc skin was very notably decreased and was also more restricted to the upper dermis (fig 2B).

FACS ANALYSIS OF CULTURED FIBROBLASTS

Figure 3 shows representative histograms of the surface expression of NEP, APN and DPPIV. A minimum of at least 14 individual different observations were carried out for each antigen, from which the average fluorescence intensities (AFIs) were calculated and the standard deviations and data analysed using the Mann-Whitney U test. Nearly all fibroblasts were found to express NEP (fig 3A) and APN (fig 3B) with a normal distribution of fluorescence values. Although the SSc cells had a slightly smaller concentration of NEP compared with the normal cells (AFI 36.1 (SD 28.1) and 47.5 (26.8), respectively), APN expression was the same in both types of cell (AFI 397 (116) and 401 (111), respectively). In marked contrast, the concentration of DPPIV in the SSc cells was, on average, significantly less than that in the normal fibroblasts (AFI 25 (16) and 47 (31), respectively) ($p < 0.001$). The particular experiment shown in figure 3C, in which the SSc cells expressed only very small concentrations of DPPIV, also reveals that frequently this enzyme was not uniformly

Figure 1 Immunoperoxidase staining of APN in normal (A) and SSc (B) skin. A: Antigen is detected in fibroblast like cells in the papillary and deep dermis (small arrows) and in association with blood vessels (large arrows). B: APN labelling intensity is similar in to that seen in the normal skin and is also localised in fibroblast like cells (small arrows) and blood vessels (large arrow). However, in this SSc skin, the staining is restricted predominantly to the upper dermis. Note the extensive collagen deposition (open arrows) in the lower dermis of the SSc skin. Magnification $\times 200$.

distributed among the cells, but was clearly differentially expressed between two distinct subpopulations of fibroblasts. In more than half the experiments carried out, fibroblasts which expressed large amounts of DPPIV were found to constitute the major proportion of normal cells, whereas the population which expressed small amounts of DPPIV was the major fibroblast subset in SSc cultures.

While some differences in NEP expression between normal and SSc cells were apparent during the early growth of the fibroblasts in tissue culture (AFI 55 and 38, respectively) (fig 4A), the concentrations of this antigen became more similar at later passages (AFI 40 and 30, respectively). In contrast, the relatively small average concentration of DPPIV measured in early cultures of SSc fibroblasts (AFI 21

Figure 2 Immunoperoxidase staining for DPPIV in normal (A) and SSc (B) skin. A: Enzyme is present in fibroblast like cells throughout the tissue (small arrows) and is not associated with the blood vessels (large arrows), as is APN in figure 1A. B: DPPIV staining is less intense and much more sparsely distributed in fibroblast like cells (small arrows) and blood vessels (large arrow). Magnification $\times 200$.

Figure 3 Flow cytometry analysis of NEP (A), APN (B) and DPPIV (C) in normal (—) and SSc (---) dermal fibroblasts. --- = Control cells stained with secondary antibody only. In the particular experiment shown in (C), note the presence of subpopulations of cells which express DPPIV in very small (arrowheads) and large (arrows) concentrations. These distinct subsets were observed in more than half the experiments.

compared with 59 in normal cells) increased somewhat at later passage, but still remained notably smaller than that of the normal cells (AFI 30 in SSc cells compared with 44 in normal fibroblasts) (fig 4B). In more than half the experiments these average concentrations reflected clear differences in the proportion of fibroblast subsets which had high and low DPPIV concentrations (as shown in figure 3C above). APN concentrations were unaffected on extended passage (data not shown).

Discussion

In this study, we have examined the expression and distribution of three cell surface peptidases in normal and SSc skin. Although NEP (CD10) was not detected by immunoperoxidase staining of cryostat sections of either SSc or control skin, APN and DPPIV (the CD antigens 13 and 26, respectively) were

Figure 4 Changes in NEP (A) and DPPIV (B) expression in cultured fibroblasts. A: The small difference in NEP expression in early passage (three to six) cultures of SSc and normal cells was also apparent in fibroblasts cultured for extended periods (eight to 12 passages). In contrast, although the expression of DPPIV in early passage cultures of SSc cells increased at later passage, it nevertheless remained substantially less than that in normal cells (B). Each point represents one measurement and the bar indicates the average antigen concentration of all the experiments.

present on fibroblast like cells in both tissues. However, the level of expression of DPPIV was substantially lower in SSc skin compared with that in normal dermal biopsy material. The localisation of all the ectoenzymes was far more evident in the area of the papillary dermis in the SSc skin than in control sections, possibly because excessive collagen deposition in the affected skin limited the number of fibroblast like cells in the lower dermis and thus restricted the fibroblast associated peptidases to the upper dermis.

The expression of the three ectoenzymes was also examined in cultured fibroblasts derived from the SSc and normal dermal biopsies. Surprisingly, FACS analysis showed that NEP was present in both SSc and normal cells grown in tissue culture, despite the apparent absence of this peptidase *in vivo*. While this discrepancy may have arisen because of the selective sensitivity of the antigen to the fixation procedure used for the cryostat tissue sections, it could also reflect downregulation resulting from the depletion, during growth *in vitro*, of naturally occurring inhibitory

substances, or the influence of stimulatory factors in the culture medium. In contrast to NEP, however, the other zinc protease, APN, was expressed in the normal and SSc dermal tissues in addition to the cultured fibroblasts.

The serine protease, DPPIV, was found to be expressed at a substantially lower level in SSc compared with control cells. The differential expression seen in vitro was consistent with our observations in vivo and was also maintained on prolonged subculture of the fibroblasts. This peptidase, which activates precursor forms of substance P and promelittin,²³ has also been shown to cleave Gly-Pro bonds in the synthetic substrate Gly-Pro-pNA.²⁴ Since collagens contain a high proportion of repeating Gly-Pro-X amino acid triplets,²⁵ it is possible that DPPIV activity may also extend to such major ECM components. Our finding that this peptidase is markedly downregulated in SSc could therefore be fundamental in contributing to the excessive accumulation of the ECM in this disease. It is of interest that DPPIV and APN are both upregulated in fibroblasts in psoriasis and rheumatoid arthritis,²⁶ in which matrix breakdown is an important part of the disease process.²⁷⁻²⁸

In addition to its importance as a proteolytic enzyme involved in ECM turnover, DPPIV has also been reported to participate directly in cell-matrix interactions by binding to collagen type I and fibronectin.²⁰⁻²⁹⁻³⁰ This adhesion function of DPPIV could thereby also have a contributory role in the abnormal accumulation of collagen and other matrix proteins in SSc skin, which the present study shows to have markedly lower peptidase concentrations compared with normal dermal tissue.

Another aspect of SSc pathology is the increase in ICAM-1 expression by dermal fibroblasts both in vivo and in vitro, which is at least partly responsible for the enhanced interaction with T lymphocytes observed in this disease.¹¹⁻³¹ Although the full range of biological substrates of DPPIV is not yet known, the downregulation of this peptidase in SSc fibroblasts could result in decreased proteolytic clipping, leading to greater concentrations of surface antigens such as ICAM-1. While the precise role of DPPIV in adhesion molecule shedding is not yet known, it is notable that the expression and function of the peptidase itself may be regulated by a shedding process, as suggested by the recent finding of a circulating, 'soluble' form of this antigen in human plasma.³²

DPPIV (CD26) is also known to be involved in the activation and proliferation of T cells, primarily of the memory (CD45RO) phenotype,³³⁻³⁷ and to be expressed by some B cells.³⁸⁻³⁹ Downregulation of the peptidase in T cells has previously been reported in immune deficient HIV infected patients³⁷ and may have a role in adenosine deaminase associated severe combined immunodeficiency disease in humans.⁴⁰ Although DPPIV expression in lymphocytes from SSc patients remains to be investigated, the results of the present study

suggest that its downregulation in fibroblasts, possibly in the same abnormal subpopulation which expresses large amounts of ICAM-1,¹¹ could play a fundamental part in the control of cellular interactions and the pathophysiology of SSc.

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