Human epidermal growth factor for the stratification of synovial lining layer and neovascularisation in rheumatoid arthritis

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SUMMARY Immunohistochemical study showed selective localisation of human epidermal growth factor (hEGF) to the synovial lining layer. Although the synovial lining layer of the rheumatoid, osteoarthritic, and traumatic joints was hEGF positive, hEGF staining was especially dense at the rheumatoid synovial lining layer; the staining increasing linearly according to the degree of stratification of the lining layer ($r=1$). Human epidermal growth factor was ultrastructurally localised to cytoplasm, especially to rough endoplasmic reticulum, of the synovial lining fibroblast-like (type B) cell. Only the cell surface of macrophage-like (type A) cells was hEGF positive. When different histological variables were compared with each other, a positive correlation was found between hEGF staining of the synovial lining layer and the degree of neovascularisation of rheumatoid synovium ($r=0.72$). Although some lymphocytes were weakly hEGF positive, neovascularisation did not correlate with the extent of lymphocyte infiltration or of hEGF staining of lymphocytes. Lymphocyte infiltration or hEGF staining of lymphocytes did not correlate with hEGF staining of the synovial lining layer, whereas the lymphocyte infiltration correlated positively with the extent of perivascular accumulation of lymphocytes ($r=0.89$). These findings suggest that (a) hEGF is synthesised by and secreted through endoplasmic reticulum and Golgi apparatus from the synovial lining type B cells; (b) hEGF is at least partially responsible for the pathogenesis of stratification of the rheumatoid synovial lining layer, and perhaps of neovascularisation of the rheumatoid synovium, whereas it is not responsible for lymphocyte accumulation to the rheumatoid synovium.

Stratification of the synovial lining layer^{1 2} and periarticular osteoporosis^{3 4} are both early, characteristic manifestations of rheumatoid arthritis, but few detailed studies have been carried out to elucidate their pathogenesis. In particular, information about the factor(s) responsible for the stratification of the synovial lining layer is not available. In this study we have focused on human epidermal growth factor (hEGF), which is a 53 amino acid polypeptide essential for cell growth in a variety of tissue.⁵ It stimulates bone resorption in neonatal mouse calvaria in vitro,⁶ and specific receptors for

hEGF are present in murine and human bone tissues.⁷⁻⁹

As synovial lining cells express HLA-DR antigen on their surface,¹⁰⁻¹³ and synovial adherent cells are indeed immunologically active, presenting antigen to T cells,¹⁴⁻¹⁶ it is expected that synovial lining cells play an important part in initiating the immune response in the synovial membrane, and in this way may contribute to the pathogenesis of chronic synovitis. Observation that the stratification of synovial lining layer represents the early, characteristic manifestation of the disease^{1 2} supports this possibility. There are, however, still questions about the origin and functional characteristics of both the synovial lining macrophage-like cells (type A) and fibroblast-like (type B) cells.¹⁷⁻¹⁹ It remains uncertain

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whether the synovial lining type A and B cells are mutually transitional or not,^{13 19-23} though at present it appears likely that synovial type A cell is of bone marrow origin.^{24 25} It is unclear also whether synovial lining type B cells are similar to fibroblast or fibroblast-like cells situated in the sublining region of synovium.^{19 21 26 27}

The purpose of our study was to characterise synovial component cells based on their content of hEGF, by using immunohistochemistry. The results show that hEGF synthesised by the synovial lining type B cell may be a potent factor in the stratification of synovial lining layer and perhaps the neovascularisation of rheumatoid synovium. The selective localisation of hEGF to the synovial lining layer may facilitate exact sorting of this population of cells.

Materials and methods

SYNOVIAL SPECIMENS

Samples of synovia were obtained during joint surgery from 20 patients with definite or classic seropositive rheumatoid arthritis,²⁸ one with osteoarthritis, and three with normal traumatic joints. Both active and inactive rheumatoid tissues were included. Cartilage-pannus junction specimens were selected only from active cellular pannus of three patients.¹⁹ Tissue specimens were fixed in 2% paraformaldehyde, 0.075 M lysine, and 0.01 M sodium periodate solution overnight at 4°C.^{29 30}

IMMUNOHISTOCHEMISTRY OF hEGF

For electron microscopy specimens cut in small pieces were washed with phosphate buffered saline pH 7.2 for at least 24 hours and reacted with rabbit anti-hEGF antisera at 4°C overnight. Anti-hEGF antisera were raised by immunising rabbits with hEGF, which was highly purified according to Cohen and Carpenter.^{7 31 32} Its specificity has been confirmed by us and by others (YH-1 in Ref 33) using radioimmunoassay against a panel of structurally related peptides, including mouse epidermal growth factor and platelet derived growth factor.³²⁻³⁴ Tissue specimens were washed with phosphate buffered saline for at least 24 hours, then reacted with the horseradish peroxidase conjugated IgG fraction of goat polyvalent antirabbit IgG F(ab)₂ antibody (Cappel Laboratories, Downingtown, PA) at 4°C overnight. The specimens were washed for 48 hours, then reacted with diaminobenzidine-H₂O₂ solution for 30 minutes at room temperature, by a modification of the Graham and Karnovsky staining method, to minimise endogenous peroxidase reactivities.³⁰ Specimens were then fixed with 1% OsO₄ for one hour at 4°C, dehydrated in graded alcohol, and embedded in Epon 812. Sections 100 nm thick

were cut with an LKB ultramicrotome and examined for peroxidase staining under Hitachi HS-9 electron microscope without counterstaining. Horseradish peroxidase staining was examined taking into account the penetration limit of the horseradish peroxidase-immunoglobulin complex, based on previous results.³⁵

Tissue specimens fixed in the same manner were also examined under light microscope to obtain a wide view of them and to minimise bias due to observation of a restricted tissue field. Tissue specimens, cut approximately 4 mm in thickness, were embedded in Tissue-Tek (Lab-Tek Products, Naperville, IL) and snap frozen in isopentane, which was precooled in a dry ice-acetone bath. A thin section, 7 µm in thickness, was cut and mounted on glass slides. It was considered that sufficient penetration of the horseradish peroxidase-immunoglobulin macromolecular complexes was obtained in a tissue section as thin as 7 µm thick.³⁵ The specimen was reacted with rabbit anti-hEGF antisera at room temperature for 30 minutes and then with horseradish peroxidase-goat antirabbit IgG for 30 minutes. After reacting with diaminobenzidine-H₂O₂, the tissue specimen was examined under photomicroscope without OsO₄ treatment.

To confirm the specificity of hEGF staining tissue specimens were allowed to react either with non-immune normal rabbit serum samples or rabbit anti-hEGF antisera preabsorbed with recombinant hEGF (GE002, Cosmobio Co Ltd, Tokyo, Japan). For preabsorption the amount of hEGF calculated from the results of radioimmunoassay was precoated on a Falcon No 3037 (Beckton-Dickinson and Co, Oxnard, California) Petri dish. Anti-hEGF antisera were then passed through these Petri dishes three times, each passage consisting of overnight incubation at 4°C in a humidified atmosphere. The supernatant thus obtained was used for hEGF staining. Anti-hEGF antisera passed through uncoated Petri dishes similarly three times were used as a positive control. Different histological variables, measured semiquantitatively with an arbitrary scale, were statistically compared with each other.³⁶

Transforming growth factor β purified from human platelets was purchased from Collaborative Research Inc, Lexington, MA, and human synthetic transforming growth factor α from Bachem Fine Chemicals Inc, Torrance, CA, respectively. The specificity of the antibody was examined by radioimmunoassay.³²

Results

When hEGF staining was examined in a light microscope it was found to be localised almost

Fig. 1 Immunohistochemistry of rheumatoid synovium. (a) A frozen section, 7 μ m thick, stained with rabbit antihuman epidermal growth factor (anti-hEGF) antisera previously passed three times through uncoated Petri dishes, followed by staining with the horseradish peroxidase conjugated IgG fraction of goat polyvalent antirabbit IgG F(ab)₂ antibody. Localisation of antibody is visualised by diaminobenzidine reaction. Note that the stratified synovial lining layer is hEGF positive. (b) Similar section stained with rabbit anti-hEGF passed three times through hEGF coated Petri dishes. Note the absence of hEGF staining. (c) A section stained with non-immune normal rabbit sera as a control. (d) Another rheumatoid section stained with untreated rabbit anti-hEGF antisera. Note the intense hEGF staining on the synovial lining layer. Human epidermal growth factor is also weakly positive in the cells of sublining synovium. (e) A section similar to (d), stained with non-immune normal rabbit sera.

Fig. 2 Left side: microscopic view of frozen section of traumatic synovium stained with rabbit antihuman epidermal growth factor (anti-hEGF) and horseradish peroxidase-antirabbit IgG antibodies similarly to Fig. 1d. Sparsely distributed synovial lining cells are hEGF positive.

Fig. 3 Microscopic view of rheumatoid cartilage-pannus junction stained for human epidermal growth factor (hEGF) in a similar manner to Fig. 1d. Active cellular pannus with vessel formation is hEGF negative.

Fig. 4 Left side: microscopic view of rheumatoid synovium stained in a similar manner to Fig. 1d. Capillaries are seen infiltrating beneath human epidermal growth factor positive stratified synovial lining layer (arrow).

Fig. 5 Histological variables of rheumatoid (●), osteoarthritic (□), and traumatic joint (○) synovia are compared semiquantitatively with each other. Human epidermal growth factor (hEGF) staining of synovial lining layer positively correlates with stratification of the lining layer and neovascularisation of synovium.

exclusively in the synovial lining layer of all specimens of rheumatoid, osteoarthritic, and normal traumatic joints (Fig. 1). Figures 1a and 1d represent hEGF staining of rheumatoid synovia, in which stratified synovial lining cells were hEGF positive. The specificity of hEGF staining was confirmed by applying either non-immune normal rabbit serum samples (Figs 1c and e) or anti-hEGF antisera previously passed through coated Petri dishes (Fig. 1b). Figure 1a represents the positive control for Fig. 1b, in which the corresponding rheumatoid section was stained with anti-hEGF antisera previously passed through uncoated Petri dishes. Although the intensity of the staining was somewhat decreased by passing through uncoated Petri dishes, the specific staining was preserved (Fig. 1a). Anti-hEGF antisera were examined by radioimmunoassay and shown not to cross react with up to 1 µg of transforming growth factor α or β (not shown). Human epidermal growth factor staining was not absolutely restricted to the synovial lining layer; positive but substantially weak hEGF staining was occasionally observed in the cells of sublining synovium (Fig. 1d).

Synovial lining layer of the traumatic joints, which was expected to show normal inflammation, occasionally showed a certain degree of stratification (Fig. 2), probably because of minor reactive changes produced by trauma. Although the cells in the proliferating region of normal traumatic synovium such as Fig. 2 stained positively for hEGF, the intensity of the staining was weak compared with that of rheumatoid synovial lining layer. In addition, the non-proliferating region of normal synovium was weakly hEGF positive. In contrast, the rheumatoid synovium was densely packed with hEGF

positive cells (Fig. 1). Human epidermal growth factor staining was not seen in the rheumatoid cartilage-pannus junction that was active and cellular¹⁹ (Fig. 3). Because a tissue section as thin as

Fig. 6 Right side: microscopic view of rheumatoid synovium stained in a similar manner to Fig. 1d. A lymphocyte cluster is weakly human epidermal growth factor (hEGF) positive. Note the intense hEGF staining on the stratified synovial lining layer.

7 μ m was used to confirm the absence of staining a false negative result due to inadequate penetration of antibodies was unlikely.

When different histological variables were compared with each other a linear correlation existed between the relative amount of hEGF positive synovial lining cells and the degree of stratification of the lining layer ($r=1$) (Fig. 5). Human epidermal growth factor staining of the synovial lining layer did not correlate with the extent of lymphocyte infiltration or perivascular accumulation of lymphocytes.

Instead, there existed a positive correlation between hEGF staining of the synovial lining layer and the degree of neovascularisation in the rheumatoid synovium ($r=0.72$) (Fig. 5). Figure 4 shows that rheumatoid synovial lining layer, which is stratified and hEGF positive, is heavily infiltrated with small blood vessels.

Some, but not all of the clusters of lymphocytes of rheumatoid synovium were hEGF positive, though the intensity of the staining was considerably weaker than that of the synovial lining layer (Fig. 6). The

Fig. 7 Correlation between lymphocyte infiltration and other histological variables. The cluster of lymphocytes is either human epidermal growth factor (hEGF) positive (○) or hEGF negative (●). Lymphocyte infiltration correlates positively with perivascular accumulation of lymphocytes.

Fig. 8 Immunoelectron microscopic view of a rheumatoid synovial lining fibroblast-like (type B) cell, stained with rabbit antihuman epidermal growth factor (anti-hEGF) and horseradish peroxidase-antirabbit IgG antibodies and observed without counterstaining. Cytoplasm of the type B cell is diffusely hEGF positive. Human epidermal growth factor is localised to rough endoplasmic reticulum (inset). Staining by hEGF is weak in the area of Golgi apparatus (arrow) and absent in mitochondria (inset, arrow head).

Fig. 9 Another immunoelectron microscopic view of a rheumatoid synovial lining type B cell. (a) Human epidermal growth factor (hEGF) is positive in the cytoplasm containing many dilated rough endoplasmic reticula. (b) A control section stained with non-immune normal rabbit serum samples. Note the absence of hEGF staining.

Fig. 10 Immunoelectron microscopic view of a rheumatoid synovial lining macrophage-like (type A) cell, stained similarly to Fig. 8. The cell surface of a type A cell is partially human epidermal growth factor (hEGF) positive. Control section stained with non-immune normal rabbit serum samples is hEGF negative (inset).

extent of lymphocyte infiltration correlated positively with perivascular accumulation of lymphocytes ($r=0.89$), whereas hEGF staining of lymphocytes did not correlate with the degree of neovascularisation in the rheumatoid synovium (Fig. 7).

Under electron microscope hEGF was localised to the cytoplasm of synovial lining fibroblast-like (type B) cells (Figs 8 and 9). Human epidermal growth factor staining was ultrastructurally localised to rough endoplasmic reticulum, sparing mitochondria, of the cell (Fig. 8). Staining on Golgi apparatus was less obvious. The fibroblast-like cell in the sublining synovium was also hEGF positive, but the intensity of the staining was very weak as compared with that of the synovial lining fibroblast-like cell. In contrast, hEGF staining was observed only on the cell surface of synovial lining macrophage-like (type A) cells (Fig. 10).

Discussion

Human epidermal growth factor and transforming growth factor α display a 35% homology in their amino acid sequence, and biological effects of these factors in vitro are often indistinguishable³⁷⁻³⁹. Both substances share the same epidermal growth factor receptor for their biological signal transduction. In this study we confirmed by radioimmunoassay that anti-hEGF antisera did not cross-react with transforming growth factors α or β . In a tissue section hEGF treatment effectively absorbed the hEGF staining.

Our study shows that hEGF is specifically localised to the synovial lining layer of rheumatoid, osteoarthritic, and normal traumatic joints. In particular, hEGF staining was dense at the rheumatoid synovium, the staining increasing linearly according to

the degree of stratification of the synovial lining layer. In addition, the ultrastructural localisation of hEGF to the cytoplasm, especially to rough endoplasmic reticulum and Golgi apparatus of the cell, fulfils the morphological criteria of synthesis and secretion of hEGF by the synovial lining fibroblast-like (type B) cell. Because only the cell surface of macrophage-like (type A) cells was also hEGF positive we may speculate that hEGF secreted from a type B cell stimulates a type A cell and in this way contributes to the stratification of synovial lining layer. The synthesis and secretion of hEGF through rough endoplasmic reticulum and Golgi apparatus as shown in this study are compatible with the established pathway for other secretory proteins.⁴⁰ Because hEGF staining was weak in the Golgi apparatus, however, it appears that not all the synthesised hEGF is actually secreted. This may be exemplified by the illustration of a kind of frustrated secretion by the type B cell as in Fig. 9. Together these findings suggest that hEGF is at least partially responsible for the pathogenesis of stratification of the synovial lining layer in rheumatoid arthritis. It is also possible that one or more factors other than hEGF operate in concert with hEGF in this process as hEGF is a progression factor for the cell proliferation.

There was a positive correlation between hEGF staining of the synovial lining layer and the degree of neovascularisation in the rheumatoid synovium. Neovascularisation, however, showed no correlation with the extent of lymphocyte infiltration or of hEGF staining of lymphocytes. Lymphocyte infiltration or hEGF staining of lymphocytes also did not correlate with hEGF staining of the synovial lining layer, whereas the lymphocyte infiltration correlated positively with the extent of perivascular accumulation of lymphocytes. These findings indicate that factor(s) other than hEGF must be responsible for attracting lymphocytes to the rheumatoid synovium. It appears that hEGF as expressed on lymphocytes is the result of in situ activation of lymphocytes rather than derived from the synovial lining layer. The results also suggest that hEGF synthesised in the synovial lining layer could be responsible for the development of neovascularisation in the rheumatoid synovium, whereas the lymphocytes are clearly not responsible for neovascularisation. Although this finding needs confirmation, the result is compatible with recent results on the angiogenic property of hEGF.⁴¹

The differential localisation of hEGF in type A and B cells found in this study is further evidence for the difference between synovial lining fibroblast-like (type B) and macrophage-like (type A) cells. The synovial lining type B cells are also distinct from

fibroblasts situated in the sublining synovium because the amount of hEGF stained in the synovial lining type B cell was distinctly greater than that in the sublining fibroblasts, even when examined in the same tissue section. As both types of cells more or less synthesise hEGF, however, it is still possible that the synovial lining fibroblast-like (type B) cell is derived from sublining fibroblasts or fibroblast-like cells by their transformation, as suggested by Fassbender *et al.*²¹ If this is the case it appears that the synthesis of hEGF may somehow be enhanced in contact with synovial fluid components. As a result of these findings we propose that synovial lining type A and B cells could now be reclassified based on their content of hEGF. Exclusive localisation of hEGF to the synovial lining layer as found in this study enables us to sort the cells of the synovial lining layer selectively. The lack of specific markers for the synovial lining layer has long been a major obstacle in this field of study.

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