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Localization of vascular endothelial growth factor in synovial membrane mast cells: examination with "multi-labelling subtraction immunostaining"

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Abstract Mast cells are believed to play a novel part in the development of destructive synovial pannus in rheumatoid arthritis (RA). This study was undertaken to investigate the localization of vascular endothelial growth factor (VEGF) in the synovial membrane using a unique immunostaining technique. Synovial specimens of RA patients were examined immunohistochemically and were compared with specimens from non-RA controls. Multi-labelling subtraction immunostaining, a modification of double- and triple-labelling immunostaining, revealed that the VEGF-positive cells were identical to tryptase-positive cells (mast cells). No other cell types were found to be positive for VEGF. The synovium of RA patients showed a larger number of VEGF-positive mast cells than that of non-RA controls ($P<0.001$). The study suggests that mast cell-derived VEGF may contribute to the development of synovial pannus in RA.

Key words Mast cells · Rheumatoid arthritis · Synovium · Vascular endothelial growth factor

Introduction

Mast cells have a well-established role in the immediate hypersensitivity reaction and chronic inflammation. Otherwise, their functions were and are mysterious. They are scattered in the connective tissues throughout the human body, and increase in their numbers has been implicated in various pathologic conditions, such as vasospasm [5, 7, 10], atherosclerosis [3, 10, 11], formation of varicose veins in the lower limbs [17], pulmonary fibrosis [16], liver fibrosis [15], and rheumatoid arthritis (RA) [1, 4, 13].

Vascular endothelial growth factor (VEGF) is thought to have a mechanistic role in the development of synovial pannus in RA, which it exerts by promoting vascular permeability and angiogenesis [6, 9, 14]. Synovial fluid in RA has a high titre of VEGF [6, 9]. Generally, the sources of VEGF have been considered to be macrophages, fibroblasts, vascular smooth muscle cells and synovial lining cells [6, 9, 14]. However, in our study, the synovial membrane mast cell was found to be the major source of this factor.

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Materials and methods

Synovial tissue specimens were prepared from the surgical biopsy specimens collected for histopathological examination in our hospital from 1995 to 1996. Only specimens with an adequate amount of synovial tissue were used in this study, and those that were small or decalcified were omitted. All ten patients had been clinically diagnosed as having RA, based on the criteria published by the American Rheumatism Association (1987), which were revised criteria for the classification of rheumatoid arthritis [2]. Ten tissue samples from these patients were the experimental subjects, in which a histological diagonsis of chronic synovitis was made. Synovial membranes with non-RA [trauma, $n=7$ and osteoarthritis (OA), $n=8$] were also prepared as controls.

Formalin-fixed paraffin-embedded specimens were used for staining with haematoxylin-eosin (H&E) and toluidine blue (pH 7.2), and for immunostaining. Immunohistochemistry was performed on each paraffin section using antibodies (Abs) and Histofine SAB-PO and/or SAB-AP kit (streptavidin–biotin–peroxidase complex or -alkaline phosphatase complex kit: Nichirei, Tokyo) in a routine stepwise manner. Primary Abs used in this study were antitryptase Ab (Dakopatts, Glöstrup, Denmark, working dilution ×150, Ig class IgG1), anti-VEGF Ab (R&D System, Minneapolis, Minn., working dilution ×200, Ig class IgG2b), anti-human macrophage Ab (CD68, Dakopatts, working dilution ×100, Ig class IgG3), and anti-α-smooth muscle actin Ab (Dakopatts, working dilution ×300, Ig class IgG2a). All Abs were monoclonal. A con-

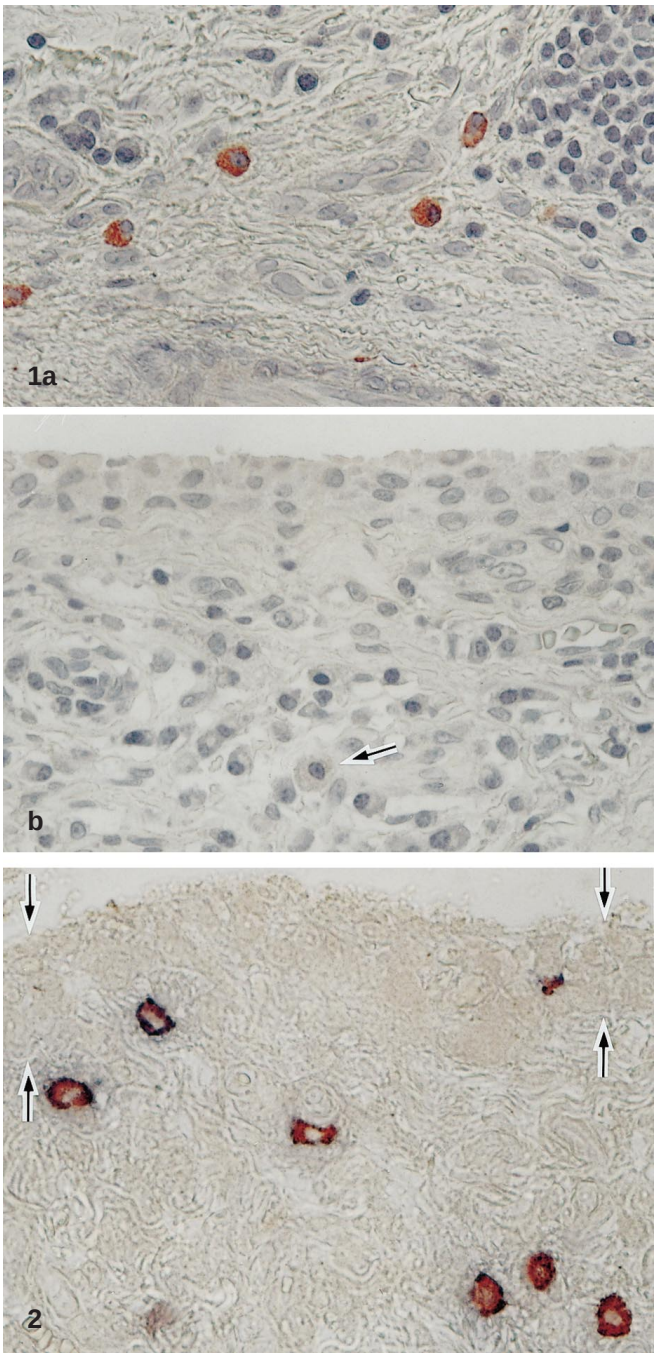


Fig. 1 a Immunostaining for VEGF. VEGF is positive (red) in the cytoplasm of scattered cells with granular staining pattern. **b** Control. No specific deposits are observed. *Arrows* indicate mast cells. Counterstain, haematoxylin, ×350

Fig. 2 Double-labelling immunostaining for VEGF (positive; *red*) and tryptase (positive; *blue*). *Red* of VEGF overlaps with *blue* of tryptase. Accurate evaluation is difficult. Synovial epithelium (*between arrows*) is negative for both VEGF and tryptase. ×350

trol study to determine the specificity of anti-VEGF Ab was accompanied, using IgG2b of nonimmune mouse (Dakopatts) with the same concentration of anti-VEGF Ab.

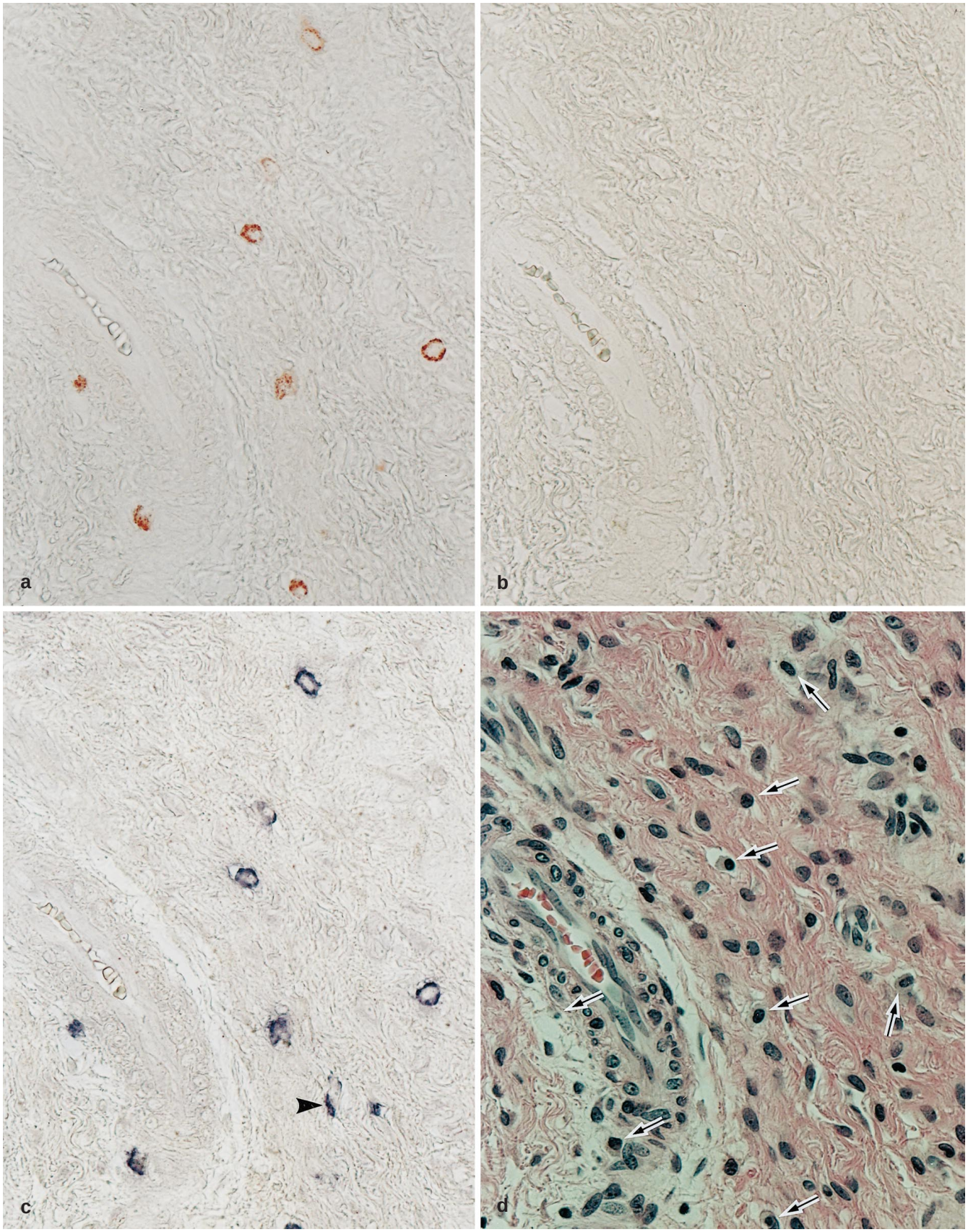
Double-labelling immunostaining was carried out to identify the cells expressing VEGF, based on the standard method with minor modifications [8]. Multi-labelling subtraction immunostaining was performed in the following stepwise fashion. After routine immunostaining for VEGF (first immunostain) using the SAB-PO kit, the slide was treated with 3-amino-9 ethylcarbazole (Sigma, St. Louis, Mo.) to produce specific deposits. The slide was immediately mounted with a coverglass, using an aqueous mounting medium (Biomeda, Foster City, Calif.). Within 20 min, a microscopic photograph was taken (Fig. 3a). Then the slide was immersed in phosphate-buffered saline containing no calcium or magnesium (PBS; pH 7.2) and was gently shaken manually until the coverglass came off. After removal of the coverglass, the slide was meticulously washed with PBS and then immersed in 99.8% methyl alcohol for 10 s to eliminate the positive deposits of the first immunostain (Fig. 3b), followed by thorough rinsing with PBS. It was then incubated with anti-tryptase Ab using the SAB-AP kit (second immunostain). At the end of the second immunostain, the specific deposits were developed by fast blue (Nichirei) and the slide was remounted using the medium (Biomeda). The exact area on the same slide glass was compared with the first immunostain (Fig. 3c). The coverglass was removed again by washing in distilled water and the slide was then immersed in dimethylbenzene for 20 s to eliminate the positive deposits of the second immunostain. Finally the slide was stained with H&E after thorough washing with distilled water (Fig. 3d).

To determine the numbers of VEGF-positive cells and mast cells, 10 inflamed RA and 15 non-RA specimens were immunostained with anti-VEGF Ab and antitryptase Ab in serial sections. More than 10 mm^2 of microscopic fields, which were equal to 56 high-power fields (×400), were examined on each section. The numbers of tryptase- and/or VEGF-positive cells were evaluated and expressed as mean±standard deviation per 0.8 mm^2. The RA group was statistically compared with the non-RA groups by using the unpaired two-tailed *t*-test. Similarly, in half of all 10 RA specimens, the numbers of VEGF-positive cells and total mast cells were compared between the inflamed area and the apparent fibrotic scar region.

Results

Immunoreactivity against VEGF was found to be limited to large round cells with abundant cytoplasm (Fig. 1). Double-labelling immunostaining suggested that VEGF-positive cells also expressed tryptase, but did not provide confirmation (Fig. 2). In multi-labelling subtraction immunostaining, VEGF-positive cells were clearly shown to be positive for tryptase and were regarded as mast cells (Fig. 3a–d). Vascular smooth muscle cells, macrophages, and synovial lining cells were all negative for VEGF. VEGF-positive cells were observed in both RA and non-RA specimens; however, in RA they were present in significantly higher numbers (inflamed areas 36.9±13.5; fibrotic areas 24.3±12.3) than in non-RA specimens (OA 15.2±6.9, trauma 12.1±6.1; Fig. 4). In RA, VEGF-positive cells were concentrated in the areas where there were many vascular channels, mild fibrosis,

Fig. 3a–d Multi-labelling subtraction immunostaining in the exact area on the same slide. **a** First immunostain for VEGF (positive; *red*). **b** After subtraction by treatment with methyl alcohol. The positive deposits of the first immunostaining are completely extinguished. **c** Second immunostaining for tryptase (positive; *blue*). *Arrowhead* indicates the cytoplasm of VEGF-negative mast cell. **d** H&E stain. It is difficult to find mast cells in H&E stain. There is a small blood vessel showing no immunoreaction for VEGF in smooth muscle cells and endothelial cells. *Arrows* indicate mast cells. ×400 ▶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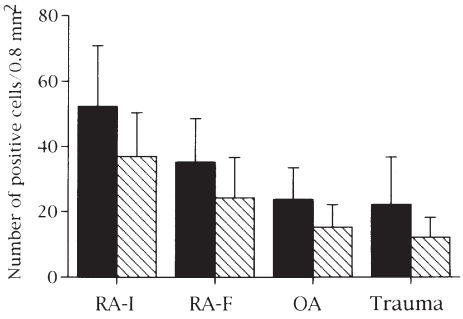

Fig. 4 Quantitative analysis of mast cells (*closed bars*) and VEGF-positive cells (*hatched bars*). Numbers of mast cells and VEGF-positive cells in RA are higher than those in OA ($P<0.001$) and trauma ($P<0.001$). The difference between the numbers of mast cells and VEGF-positive cells in *RA-I* (inflamed area) and *RA-F* (fibrotic scar area) is also statistically significant ($P<0.001$)

and mild lymphocytic infiltration beneath the densely inflamed lesion; such areas were regarded as "inflamed" in this study. The proportions of VEGF-positive mast cells to total mast cells were 70.6% in inflamed RA, 69.0% infibrotic RA, 63.9% in OA, and 54.8% in trauma, respectively.

Discussion

The synovial membrane in RA is characterized by villous proliferation of synovial epithelium, an increase of small blood vessels, fibrosis, and a varying degree of infiltration of inflammatory cells, including lymphocytes, macrophages, and mast cells. In normal human synovium, a few mast cells may be found in the perivascular region. In RA, their number is significantly increased, although this increase is not specific to RA [1, 4, 13]. Mast cells are also increased in the synovial fluid in various other inflammatory types of arthritis, in parallel with an increase in fluid histamine level [12]. In addition, synovial fluid contains high concentrations of VEGF [6, 9], which, we believe, implies that synovial membrane mast cells release VEGF.

We have shown here that mast cells are the major source of VEGF and that RA has an elevated number of VEGF-positive mast cells. The study directly links the previous findings [6, 9, 12] and concludes that mast cell-derived VEGF contributes to the inflammatory processes in RA. We have used and introduced a unique immunohistochemical approach to obtain the best identification from double-labelling immunostaining. Since tryptase and VEGF were simultaneously expressed in mast cell granules by the conventional double-labelling immunostaining (Fig. 2), they were difficult to distinguish from one another. In contrast, multi-labelling subtraction immunostaining seems to be much more useful in identifying cells expressing numerous factors.

Theoretically, if the method is repeated several times with different primary Abs, many kinds of factors can be examined successively in one type of cell on the same tissue section or culture dish.

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