Virchows Arch (1998) 433:55–61

ORIGINAL ARTICLE

Katsunori Shigehara · Noriharu Shijubo
Michio Hirasawa · Shosaku Abe · Toshimitsu Uede

Immunolocalization of extracellular matrix proteins and integrins in sarcoid lymph nodes

Received: 2 December 1997 / Accepted: 19 February 1998

Abstract To improve our understanding of the role of extracellular matrix (ECM) proteins and integrins during the processes of granuloma formation in sarcoidosis, we examined the distribution of ECM proteins and the expression of integrins in sarcoid lymph nodes by immunohistochemical methods. We also examined the expression of transforming growth factor-β1 (TGF-β1), which is one of major regulators for synthesis of ECM proteins. Most ECM proteins were detected in the periphery of the granulomas in a concentric pattern, and fibronectin was diffusely detected from an early to a regressive stage. Compared with normal lymph nodes, most β1-integrin subfamilies (α1, α4, α5 and α6) were more strongly expressed on lymphocytes around the granulomas. Epithelioid cells exhibited strong expression of the α5 molecule. Fibroblasts exhibited the expression of the α2 and α5 molecules surrounding ECM proteins. The α5β1 molecule had a distribution similar to that of fibronectin. TGF-β1 was detected in epithelioid cells throughout the various evolutional stages and its expression was especially marked in mature granulomas. Interaction of fibronectin and the α5β1 molecule may have an important role in the process of formation of sarcoid granuloma. The expression of TGF-β1 may be involved in the regression of sarcoid granuloma by initiating fibrosis and atrophy of epithelioid cells.

Key words Adhesion molecule · Extracellular matrix proteins · Transforming growth factor-β1 · Granuloma formation and regression · Sarcoidosis

K. Shigehara (✉) · N. Shijubo · M. Hirasawa · S. Abe
Third Department of Internal Medicine,
Sapporo Medical University School of Medicine, South-1,
West-16, Chuo-ku, Sapporo, 060, Japan,
Tel.: (+81)-11-611-2111, ext. 3239, Fax: (+81)-11-613-1543

T. Uede
Section of Immunopathogenesis,
Institute of Immunological Science, Hokkaido University,
Sapporo, Japan

Introduction

Integrins are heterodimeric molecules that are composed of α and β chains, and they have important roles in cell–cell and cell–matrix interactions. In particular, most receptors for extracellular matrix (ECM) proteins belong to the integrin subfamily, and integrins have different specificities for ECM proteins such as collagen, fibronectin, and laminin [9, 14, 25]. In inflammation, it is very important for leukocytes to adhere to endothelial cells, to emigrate into inflammatory sites, and to remain at the affected sites. Both β1-integrins on leukocytes and β2-integrins make a large contribution to these phenomena by adhering to ECM proteins [22, 23].

In sarcoidosis, the unknown causative agent leads to the accumulation of activated $CD4^+$ lymphocytes and macrophages and to granuloma formation [5, 12, 13]. Several investigators [4, 17, 18] have reported on the distribution of ECM proteins and integrin expression in sarcoid granuloma. However, there have been few reports on these distributions in the different evolutional stages. Transforming growth factor-β1 (TGF-β1) is one of the most important cytokines for the regulation of ECM protein synthesis [15] and can influence the expression of β_1-integrins [8, 26]. Wishing to understand the role of ECM proteins and integrins in the processes of granuloma formation and regression better, we studied the distribution of ECM proteins and the expression of integrins and of TGF-β1 in sarcoid lymph nodes in each evolutional stage.

Materials and methods

Scalene lymph nodes were obtained at surgery for diagnostic purposes from 25 untreated patients whose histological findings were consistent with sarcoidosis (non-caseating epithelioid cell granulomas). Informed consent was obtained from all patients. These patients had no evidence of mycobacterial, fungal, or parasitic infection. None had a history of exposure to organic or inorganic materials known to cause granulomatous disorders. Histological findings of sarcoid lesions in scalene lymph nodes differed according to the evolutional stage. In a previous study [1], sarcoid lymph

nodes were classified into three stages according to the histological findings: (1) early stage, consisting of sinus histiocytosis or some immature granulomas (*n*=5); (2) active stage, containing a lot of mature granulomas, occasionally with polynucleated giant cells (*n*=12); and (3) regressive stage, showing fibrosis or hyalinosis in most granulomas, with degeneration and atrophy of epithelioid cells (*n*=8). Control lymph nodes were obtained from 8 patients who underwent pulmonary, gastric or colic resection for cancer. The control lymph nodes showed no abnormal findings.

Large fragments of lymph nodes were fixed with 10% formalin and embedded in paraffin for routine histological examinations. Small fragments of lymph nodes were embedded in optimal cryopreserved tissue compound (Miles, Elkhart, Ind.), snap-frozen in liquid nitrogen, and stored at –80°C until cryostat sectioning.

The antibodies used in the present study are listed in Table 1. We investigated the expression of three groups of molecules: (1) ECM proteins including collagen I, collagen III, collagen IV, fibronectin, laminin, vitronectin and tenascin; (2) cell–matrix adhesion molecules: α1, α2, α3, α4, α5, α6, αv, β1 and β4 integrin molecules; and (3) TGF-β1.

Table 1 Primary antibodies

Antigen	Antibody	Source [reference]
Integrins		
α1	TS2/7	Dr. M. Hemler [10]
α2	12F1	Dr. V. Wood [19]
α3	J134	Dr. T. Albino [7]
α4	8F2	Dr. M. Hemler [11]
α5	BIIG2	Dr. C. Damsky [27]
	SAM1	Immunotech
α6	GoH3	Dr. A. Sonnenberg [24]
αv	VRN147	Chemicon
β1	AJ2	Dr. T. Albino [3]
β4	439-9B	Dr. S.J. Kennel [16]
Extracellular matrix proteins		
Collagen I	Polyclonal	Chemicon
Collagen III	Polyclonal	Chemicon
Collagen IV	Polyclonal	Life Technologies
Fibronectin	Polyclonal	Dako
Laminin	Polyclonal	Chemicon
Vitronectin	M2	Iwaki
Tenascin	TN2	Life Technologies
TGF-β1	Polyclonal	King Brewing

An indirect immunoperoxidase technique was applied for all antibodies. To stain vitronectin and integrin subunits, 5-μm sections of frozen tissue were cut with a cryostat. After being air-dried at room temperature, sections were fixed in cold acetone for 10 min. After rehydration in phosphate-buffered saline (PBS), sections were preincubated with normal goat or rabbit serum for 30 min to remove nonspecific binding, and then incubated with primary antibody in an appropriate dilution for 60 min at room temperature. After being washed with PBS, the slides were sequentially incubated with biotin-conjugated goat anti-mouse or rabbit anti-rat immunoglobulin antibody for 30 min, followed by avidin–biotin–peroxidase complex (Vecter Laboratories, Berlingame, Calif.) for 30 min, with three fold washing with PBS for 5 min between each step. The sections were finally incubated with 0.03% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine. Slides were then washed in running tap water, counterstained with hematoxylin, and mounted in Canadian balsam.

To stain ECM proteins, except vitronectin, αv molecule and TGF-β1, formalin-fixed, paraffin-embedded sections were used. Before incubation with primary antibody, the sections were treated with 0.1% trypsin (Sigma, St Louis, Mo.) in 50 mM Tris-hydrogen chloride (pH 7.6) for 120 min at 37°C (for ECM protein staining) and with 1000 U/ml hyaluronidase (Worthington Biochemical, Frehold, N.J.) in 50 mM sodium acetate, 0.85% sodium chloride, for 20 min at 37°C (for TGF-β1 staining). Immunohistochemistry of the formalin-fixed sections was performed according to the procedure mentioned above. Biotin-conjugated goat anti-rabbit or anti-mouse immunoglobulin antibody was used as the second antibody. Normal mouse, rat, or rabbit immunoglobulin was used as a negative control. No significant reaction occurred in these cases.

Results

Normal lymph nodes

Each of the ECM proteins was distributed at various sites (paracortex, follicle and medulla) of normal lymph nodes. Capillary vessels, including high endothelial venules (HEVs), were positive for all ECM proteins except vitronectin. In paracortex, fibroblastic reticular cells (RCs) were positive for all ECM proteins, but in medulla, RCs showed negative staining for collagen IV, laminin and vitronectin. Fibrillar collagens including collagen I and collagen III were predominantly observed in

Table 2 Distribution of ECM proteins in human sarcoid lymph nodes (*RCs* reticular cells, *CI* collagen I, *CIII* collalgen III, *Fn* fibronectin, *CIV* collagen IV, *Ln* laminin, *Vn* vitronectin, *Tn* tenascin; ++ strongly stained, + moderately stained, +w weakly stained, – no staining, * partial structure stained, † concentrical distribution in periphery of granuloma)

Stage	Site	CI	CIII	FN	CIV	LN	VN	TN
	Granulomas	+w†	+w†	+†	–	–	+w†	+w†
Early	Around granulomas							
	Capillaries	+w	+w	+	+	+	+	+
	RCs	+*	+	+	+*	–	+	+
	Granulomas	+†	+†	+†	–	–	+†	+†
Active	Around granulomas							
	Fibroblasts	+w	+w	+	–	–	+	+*
	Capillaries	+w	+w	+	+	+	+	+*
	Granulomas	++†	++†	++†	–	–	++†	++†
Regressive	Around granulomas							
	Fibroblasts	++	++	++	–	–	++	++
	Capillaries	+w	+w	+	+	+	+	+

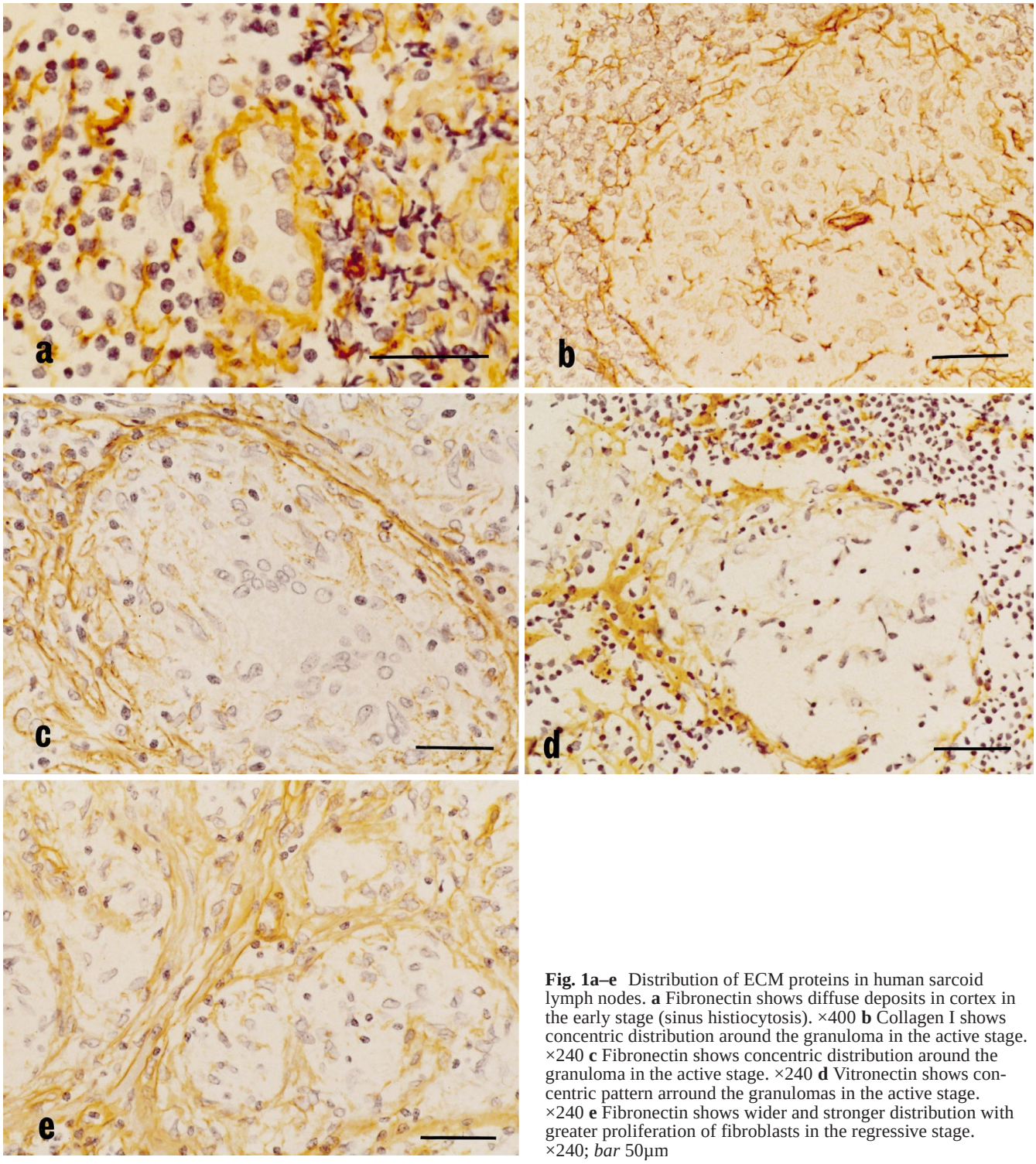


Fig. 1a–e Distribution of ECM proteins in human sarcoid lymph nodes. **a** Fibronectin shows diffuse deposits in cortex in the early stage (sinus histiocytosis). ×400 **b** Collagen I shows concentric distribution around the granuloma in the active stage. ×240 **c** Fibronectin shows concentric distribution around the granuloma in the active stage. ×240 **d** Vitronectin shows concentric pattern arround the granulomas in the active stage. ×240 **e** Fibronectin shows wider and stronger distribution with greater proliferation of fibroblasts in the regressive stage. ×240; *bar* 50μm

reticular fiber bundles. Follicular dendritic cells (FDCs) in follicles were positive only for vitronectin. Tenascin was detected in endothelial cells and some RCs.

Lymphocytes were positive for the β1 and α4 molecules, and some of them were positive for the α5 and α6 molecules. No α1, α2, α3, αv or β4 molecules were found in lymphocytes. The integrin expression of macrophages, although weak, was closely similar to that of lymphocytes. Endothelial cells, including HEVs, were positive for all integrin subunits except the α4 molecule.

TGF-β1 was weakly expressed in RCs and some macrophages.

Table 3 Expression of integrin subunits in human sarcoid lymph nodes (+ strongly or moderately stained, +w weakly stained, – no staining, * some of cells stained)

Site	α1	α2	α3	α4	α5	α6	αv	β1	β4
Within granuloma									
Epithelioid cells	–	–	–	+w	+	+w	+*	+	–
Lymphocytes	+w*	–	–	+	+	+*	–	+	–
Multinucleated giant cells	–	–	–	+w	+	+w	–	+	–
Without granuloma									
Lymphocytes	+*	–	+w*	+	+	+*	–	+	–
Macrophages	+*	–	+w*	+	+	+w*	–	+	–
Fibroblasts	–	+	–	–	+	–	+	+	–
Endothelial cells	+*	+w	+*	–	+	+	+	+	+

Sarcoid lymph nodes

Data on the distribution of ECM proteins in sarcoid lymph nodes are summarized in Table 2. In the early stage, marked angiogenesis occurred. All ECM proteins examined in this study were found to be associated with vessels and capillaries, and also with RCs, except for laminin. Fibronectin exhibited diffuse distribution with fibrillar pattern and a focal deposit in the paracortex in sinus histiocytosis (Fig. 1a). In immature granulomas fibronectin was detected in a concentric pattern in the periphery of the granulomas.

In the active stage of sarcoid lymph nodes, all the ECM proteins except collagen IV and laminin were easily detected in a concentric pattern in the periphery of the granulomas and were seen faintly with a fibrillar pattern within and around the granulomas (Fig. 1b–d). In the regressive stage of sarcoid lymph nodes all the ECM proteins except for collagen IV and laminin were more diffusely and more strongly expressed within and around the granulomas, with a more active proliferation of fibroblasts than in the active stage (Fig. 1e).

Data on the expression of integrins in sarcoid lymph nodes are summarized in Table 3. The expression of integrins was almost the same in all the various evolutional stages. In the regressive stage, most epithelioid cells showed degeneration and atrophy. Therefore, it was very difficult to clarify whether integrin expression in epithelioid cells on frozen sections was positive or not. Almost all epithelioid cells expressed the α5 and β1 molecules, while epithelioid cells in the periphery of the granulomas expressed the αv molecule. Lymphocytes within and around the granulomas expressed the α5 and β1 molecules, while lymphocytes around the granulomas expressed the α4 molecule (Fig. 2a–c). The α5 molecule was diffusely expressed in sarcoid lymph nodes, and its expression corresponded with the distribution of fibronectin. Expression of the αv molecule corresponded with the distribution of vitronectin. Expression of the α4 molecule was not related to the distribution of fibronectin.

In sarcoid lymph nodes, TGF-β1 was detected in the granulomas throughout all evolutional stages. Epithelioid cells, including polynucleated giant cells in mature granulomas in the active stage, had stronger TGF-β1 expression than those in immature granulomas (Fig. 3). In the regressive stage, degenerated and atrophic epithelioid cells had weaker expression of TGF-β1. Fibroblasts showed positive staining.

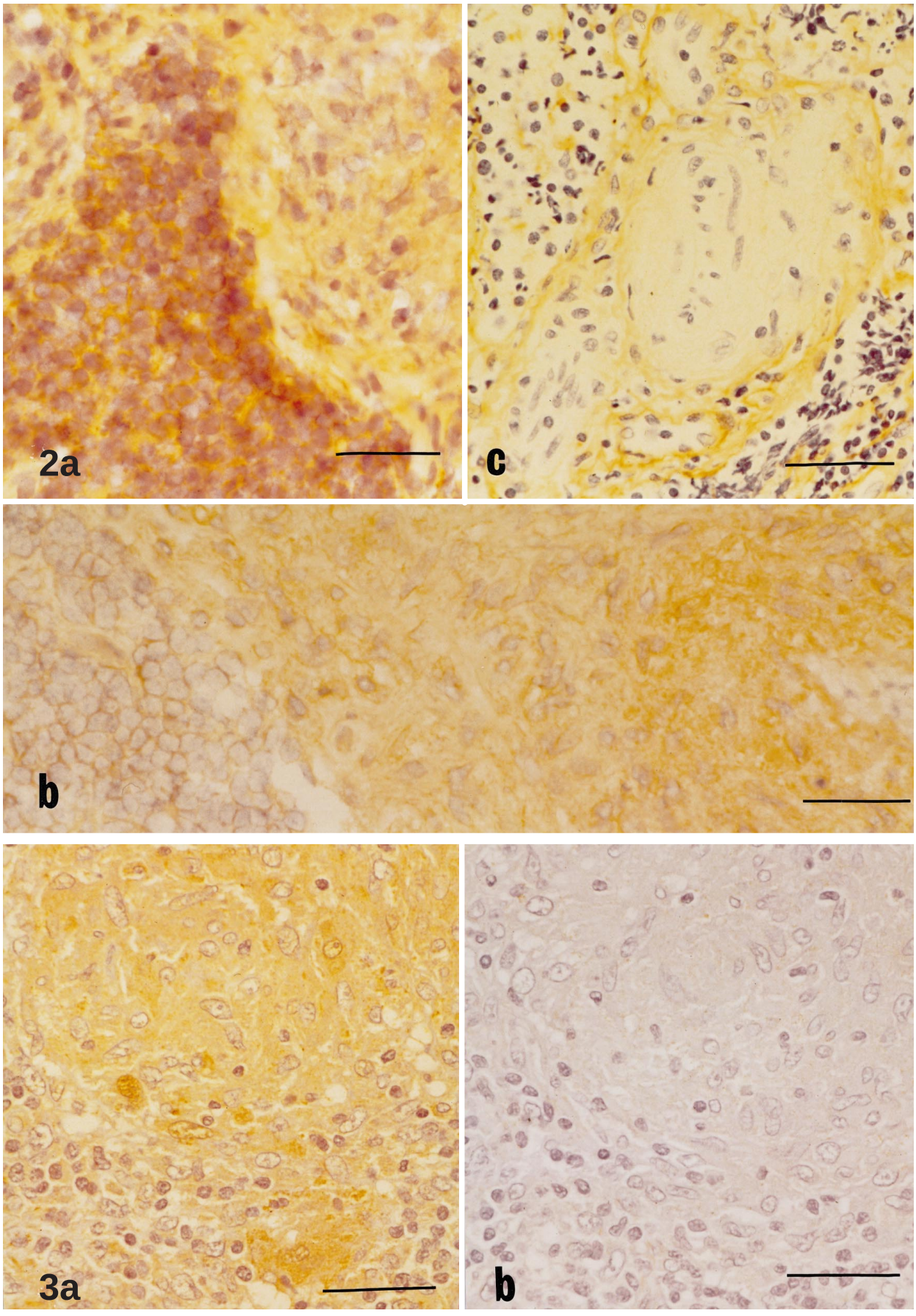
Discussion

In this study, we document the characteristic distribution of ECM proteins in sarcoid lymph nodes in each evolutional stage. Deposits of fibronectin and of vascular related collagen IV and laminin were observed at sites of angiogenesis in the early stage. Although most ECM proteins were distributed in the periphery of mature sarcoid granulomas with concentric pattern, fibronectin was most intensively stained in the active stage. In the regressive stage, most ECM proteins were diffusely distributed within and around the granulomas, with a proliferation of fibroblasts. The present results are in agreement with the results described by Peyrol et al. [18] (collagen I, III, and IV, and laminin, and fibronectin) and Chilosi et al. [4] (tenascin). To our knowledge, this is the first report on the immunohistochemical distribution of vitronectin in sarcoid granulomas.

This study demonstrates the expression of the α5β1 molecule on lymphocytes, macrophages and epithelioid cells within granulomas and expression of the α4β1 and α5β1 molecules on lymphocytes and macrophages around granulomas. The αv molecule was found on epithelioid cells in the periphery of granulomas and fibroblasts around granulomas. The expression of the α5 and αv molecules on these cells was closely associated with the immunohistochemical distribution of fibronectin and vitronectin, respectively. The expression of the α4β1

Fig. 2a–c Expression of integrins of human sarcoid lymph nodes. **a** The α4 molecule is strongly positive for lymphocytes around the granulomas. ×400 **b** The α5 molecule is positive for epithelioid cells, and positive for lymphocytes within and around the granuloma. ×400 **c** The αv molecule is positive for epithelioid cells in the periphery of the granulomas and fibroblasts. ×400; *bar* 50μm

Fig. 3a, b Expression of TGF-β1 of human sarcoid lymph nodes. **a** Epithelioid cells are diffusely positive. ×400 **b** Negative control. ×400; *bar* 50 μm


2a
c
b
3a
b

molecule is not related to the distribution of fibronectin. In sarcoid lesions, fibronectin binds to epithelioid cells on electron-microscopic examination [18]. Limper et al. [17] have found that the α5β1 molecule is located in a distribution similar to that of fibronectin in pulmonary sarcoidosis, and our result is in accordance with their paper. It has been reported that fibronectin increases cell spreading, migration and proliferation, and up-regulates production of cytokines through interaction between the α5β1 molecule and fibronectin [20]. Interaction of fibronectin and the α5β1 molecule on lymphocytes and macrophages may induce their proliferation and the production of cytokines, and may be one of the most important processes in the differentiation of cells from macrophages to epithelioid cells, resulting in sarcoid granuloma formation.

In sarcoid granulomas, fibrosis is observed in the periphery of granulomas even in the active stage, and it progresses into the inner area of granulomas. In the regressive stage, sarcoid granulomas finally disappear as a consequence of fibrosis and hyalinosis. The present study documents abundant expression of ECM proteins (collagen I and III, fibronectin, vitronectin and tenascin) in the regressive stage and expression of the α2, α5 and αv molecules of fibroblasts in sarcoid lymph nodes. It also demonstrates the staining of TGF-β1 throughout the various stages of sarcoid lymph nodes; intensive staining of TGF-β1 was found in mature granulomas. Fibroblasts in the regressive stage also exhibited TGF-β1 expression. Recently, Limper et al. [17] also reported that TGF-β1 was immunohistochemically detected in sarcoid granulomas of the lung. A number of lines of evidence support a role of TGF-β1 in tissue repair and fibrosis [7, 8, 15, 26]. TGF-β1 enhances the gene expression of ECM proteins, including fibronectin and collagen, and their corresponding cell surface receptors (the α2β1 and α5β1 molecules, etc.) in vitro. In addition, TGF-β1 suppresses the synthesis of matrix-degrading proteinases and enhances the expression of proteinase inhibitors in cultured fibroblasts. TGF-β1 also possesses immunosuppressive effects and regulates cellular proliferation [21]. Although the mechanisms of fibrosis in sarcoidosis have not been clear, TGF-β1 may have an important role in fibrosis and regression of sarcoid granulomas.

A recent study has shown that β1-integrins are involved in cell survival [2]; β1-integrins are the major transmitters of ECM-derived signals to the cells, for regulation of apoptosis. Although speculative, there may be a possibility that the change of contact between β1-integrins on the cells of sarcoid granulomas and ECM proteins induces apoptosis of these cells during the process of sarcoid granuloma regression.

The interaction between fibronectin and the α5β1 molecule may be important in granuloma formation. TGF-β1 may also be important in granuloma regression.

Acknowledgements The authors wish to thank Dr. M. Ohmichi, Y. Hiraga and M. Sasaki for supplying sarcoid scalene nodes and giving important advice on clinical and pathological data. We also wish to thank Dr. M. Hemler, Dr. V. Wood, Dr. T. Albino, Dr. C. Damsky, Dr. A. Sonnenberg and Dr. S. J. Kennel for generous gifts of antibodies.

References

1. Barrie HJ, Bogoch A (1953) The natural history of the sarcoid granuloma. Am J Pathol 29:451–470
2. Bates RC, Lincz LF, Burns GF (1995) Involvement of integrins in cell survival. Cancer Metastasis Rev 14:191–203
3. Carneross JG, Matles MJ, Bersersford HR, Albino AP, Houghton AN, Lloyd KO, Old LJ (1982) Cell surface antigens of human astrocytoma defined by mouse monoclonal antibodies: identification of astrocytoma subsets. Proc Natl Acad Sci USA 79:5641–5645
4. Chilosi M, Lestani M, Benedetti A, Montagna L, Pedron S, Scarpa A, Menestrina F, Hirohashi S, Pizzolo G, Semenzato G (1993) Constitutive expression of tenascin in T- dependent zones of human lymphoid tissues. Am J Pathol 143:1348–1355
5. Crystal RG, Bittermann PB, Rennard SI, Hance AJ, Keogh BA (1984) Interstitial lung disease of unknown cause. N Engl J Med 310:154–166
6. Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJ, Angel P, Heath JK (1987) Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J 6:1899–1904
7. Fradet Y, Cordon-Cardo C, Thomson T, Daly ME, Whitemore WF Jr, Lioyd KO, Melamed MR, Old LJ (1984) Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies. Proc Natl Acad Sci USA 81:224–228
8. Heino J, Ignotz RA, Hemler ME, Crouse C, Massagué J (1993) Regulation of cell adhesion receptors by transforming growth factor-β. J Biol Chem 264:380–388
9. Hemler ME (1990) VLA proteins in the integrin family: structures, functions, and their role on leukocytes. Annu Rev Immunol 8:365–400
10. Hemler ME, Sanchez-Madrid F, Flotte TJ, Krensky AM, Burakoff SJ, Bhan AK, Springer TA, Strominger JL (1984) Glycoproteins of 210,000 and 130,000 m.w. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. J Immunol 132:3011–3018
11. Hemler ME, Huang C, Takada Y, Schwarz L, Strominger JL, Clabby ML (1987) Characterization of the cell surface heterodimer VLA-4 and related peptides. J Biol Chem 262:11478–11485
12. Hunninghake GW, Crystal RG (1981) Pulmonary sarcoidosis. N Engl J Med 305:429–434
13. Hunninghake GW, Gadek JE, Young RC, Kawanami O, Ferrans VJ, Crystal RG (1980) Maintenance of granuloma formation in pulmonary sarcoidosis by T lymphocytes within the lung. N Engl J Med 302:594–598
14. Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25
15. Ignotz RA, Massague J (1986) Transforming growth factor beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337–4345
16. Kennel SJ, Foote LJ, Flynn KM (1986) Tumor antigen on benign adenomas and on murine lung carcinomas quantitated by a two-site monoclonal antibody assay. Cancer Res 46:707–712
17. Limper AH, Colby TV, Sanders MS, Asakura S, Roche PC, Deremee RA (1994) Immunohistochemical localization of transforming growth factor-β1 in the nonnecrotizing granulomas of pulmonary sarcoidosis. Am J Respir Crit Care Med 149:197–204

18. Peyrol S, Takiya C, Cordier JF, Grimaud JA (1986) Organization of the connective matrix of the sarcoid granuloma: evolution and cell-matrix interactions. Ann NY Acad Sci 465:268–285
19. Pischel KD, Hemler ME, Huang C, Blustein HG, Woods VL Jr (1987) Use of monoclonal antibody 12F1 to characterize the differentiation antigen VLA-2. J Immunol 138:226–233
20. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT (1992) pp125^{FAK}, as structurally distinctive protein tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA 89:5192–5196
21. Shalaby MR, Ammann AJ (1988) Suppression of immune cell function in vitro by recombinant human transforming growth factor β. Cell Immunol 112:343–350
22. Shimizu Y, Van Seventer GA, Horgan KJ, Shaw S (1990) Regulated expression and binding of three VLA (β_1) integrin receptors on T cells. Nature 345:250–253
23. Shimizu Y, Van Seventer GA, Horgan KJ, Shaw S (1990) Costimulation of proliferative responses of resting CD4$^+$ T cells by the interaction of VLA-4 and VLA-5 with fibronectin or VLA-6 with laminin. J Immunol 145:59–67
24. Sonnenberg A, Daams H, Van der Valid MA, Hilkiens J, Hilger J (1986) Development of mouse mammary gland: identification of stages in differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. J Histochem Cytochem 34:1037–1046
25. Spriger TA (1990) Adhesion receptors of the immune system. Nature 346:425–434
26. Wahl SM, Allen JB, Weeks BS, Wong HL, Klotman PE (1993) Transforming growth factor beta enhances integrin expression and type IV collagenase secretion in human monocytes. Proc Natl Acad Sci USA 90:4577–4581
27. Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH (1989) Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J Cell Biol 109:877–889