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Differential distribution of FcγRIIIa in normal human tissues and co-localization with DAF and fibrillin-1: implications for immunological microenvironments

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SUMMARY

FcγRIIIa is a cytokine-inducible IgG Fc receptor implicated in the activation of macrophages by immune complexes. Differential expression of FcγRIIIa by macrophages in different tissues may therefore modulate local immune responsiveness. FcγRIIIa expression in normal human tissues was assessed semiquantitatively using microdensitometry. Synovial intimal, serosal, alveolar, salivary gland and placental macrophages, Kupffer cells, and macrophages in mechanically stressed dermis expressed high levels of FcγRIIIa. Less consistent expression was seen in skeletal muscle and lymphoid organs. No significant expression was observed in brain, thyroid, spine, intestine, myocardium, prostate, uterus, flexor forearm dermis, uterus, or kidney. Staining for FcγRIII was also observed on extracellular matrix, and co-localized with both complement decay-accelerating factor and fibrillin-1. It is proposed that differential levels of both cellular and extracellular FcγRIIIa, by modulating the response to immune complexes, may contribute to relative tissue susceptibility to infection and autoimmune disease.

INTRODUCTION

Specialized immunological microenvironments, based on features such as vascular permeability, adhesion molecule expression and local growth factor levels are well recognized in organs such as the central nervous system, eye, skin, mucosae and gut.[1–3] This specialization is likely to be important in tissue susceptibility to infection and may also, directly or indirectly, determine tissue susceptibility to autoimmune disease.[4–6]

An important feature of an immunological microenvironment is the regulation of interactions involving immunoglobulin, complement and associated receptors and regulatory proteins. An example of such specialization is the differential expression of complement decay-accelerating factor (DAF, CD55) on resident cells in tissues such as amnion, synovium and pericardium.[7] A recent study has also indicated differential expression of the immunoglobulin receptor, FcγRIIIa (CD16a) within synovium.[6]

FcγRIIIa is an IgG Fc receptor expressed by macrophages, certain, chiefly cytotoxic, T-cell subsets and natural killer cells.[8] The FcγRIIIa α-chain is encoded by the FcγRIIIA gene.[9] The homologous FcγRIIIB gene encodes FcγRIIIb, expressed on granulocytes. The extracellular domains of the two receptors are near-identical. However, whereas FcγRIIIa has a cytoplasmic domain linked to two γ- or ζ-chains, FcγRIIIb is glycophosphatidylinositol-linked.[8] Studies on granulocytes suggest that FcγRIII-class receptors are particularly essential to the binding of dimeric immune complexes.[10]

Previous information on FcγRIIIa expression in normal tissues is difficult to interpret.[11–14] *In vitro*, FcγRIIIa is expressed by macrophages cultured for several days.[12] *In vivo*, FcγRIIIa has been consistently described on Kupffer cells, but data on other tissue macrophages are conflicting.[11–14] One study suggests that, in the absence of lesions such as tumours, expression is minimal.[14] The problem is compounded by the existence of FcγRIIIa$^+$ lymphoid cells and the failure of some studies to distinguish granulocytes (carrying FcγRIIIb). Our own study suggests that, at least in fetal tissues, FcγRIIIa expression by tissue macrophages may be highly restricted.[6]

Previous studies of FcγRIIIa expression have been qualitative. It is theoretically feasible to make quantitative observations, using scanning and integrating microdensitometry[15,16] (digital image analysis systems are less satisfactory). However, there are problems of standardization, which, in the context of biological variation, make semiquantitative analysis more realistic. Mean readings cannot be derived for populations if unstained cells cannot be identified. For this reason mean maximal microdensitometric readings of macrophage staining for FcγRIIIa for 10 randomly sampled high-power fields have been obtained.

In our previous study, it was noted that pericellular staining for FcγRIIIa in synovium frequently co-localized with staining for DAF, in a way difficult to reconcile with evidence that the two molecules were predominantly synthesized by macrophages and fibroblasts, respectively.[6] DAF is an inhibitor of complement-mediated cell lysis[17] and also of killing by natural

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killer cells.[18] DAF is expressed at high levels on cells lining body cavities.[7] DAF has also been observed in association with fibrillar matrix components, variously identified as collagens, elastin, or fibrillins.[7,19,20] The major fibrillar components of synovial intima are fibrillin-1 and collagen VI-based microfibrils.[21] The objective of the present study was to compare expression of FcγRIIIa in a wide range of normal tissues in a semiquantitative fashion and to analyse the relationship between FcγRIII, DAF and specific matrix elements.

MATERIALS AND METHODS

Tissues

Normal human tissues were obtained from surgical procedures performed at University College London Hospitals, not relating to pathology of the tissue obtained, and post-mortem (Tissue Bank, Addenbrookes Hospital, Cambridge). Normality was confirmed histologically. Tissue samples were embedded in OCT compound (Tissue Tek, Elkhart, IN), snap frozen in *n*-hexane (BDH Laboratories Supplies, Poole, UK) and stored at −80°. Two specimens were examined of each of the following tissues except where numbers are indicated in brackets: synovium (5), lung, intestine, spleen, breast, myocardium, pericardium, brain, prostate, kidney, uterus, thyroid, liver, spinal disc and enthesis, placenta, umbilical cord, flexor forearm skin, skin exposed to mechanical stress; at the elbow (1) and over a toe interphalangeal joint (1), fingernail (1), salivary gland (1), gall bladder (1), tonsil (4), bone marrow aspirate smear (1) and skeletal muscle (5).

Immunohistochemistry

Tissues were sectioned to a thickness of 5 μm at −25°, taken up onto slides, air dried for 1 hr and fixed with cold acetone (BDH Laboratories Supplies) for 10 min. Between all further steps tissues were washed in two changes of phosphate-buffered saline (PBS) for 5 min each.

Serial sections of each tissue were incubated with either a mouse monoclonal IgG1 anti-human pan-FcγRIII (3G8 clone; Cambridge Bioscience, Cambridge, UK) at 25 μg/ml (in duplicate), a mouse monoclonal IgM anti-human DAF (BRIC 128 clone; NBGRL Research products, Bristol, UK) at 14 μg/ml or a mouse monoclonal IgG1 anti-human CD68 at 10 μg/ml (EBM11 clone; Dako, High Wycombe, UK). Synovial sections were also incubated with a purified mouse monoclonal IgG1 anti-human FcγRI at 15 μg/ml (10.1 clone; gift of Dr N. Hogg, Imperial Cancer Research Fund, London, UK) and a purified mouse monoclonal IgG2b anti-human FcγRII at 12 μg/ml (IV.3 clone; gift of Dr M. Fanger, Medarex Inc, Lebanon, NH). All antibodies were diluted in PBS and incubated for 60 min at room temperature. Controls were incubated in PBS. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide in methanol. Sections were then incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako) at 40 μg/ml for 30 min followed by diaminobenzidine hydrochloride (Sigma-Aldrich, Poole, UK) for 10 min, washed in tap water, counterstained in Harris's haematoxylin (except for one of each pair stained for FcγRIII) dehydrated in alcohol, cleared in Histoclear (National Diagnostics, Atlanta, GA) and mounted in DePeX (BDH Laboratories Supplies, Poole, UK). Uncounterstained sections stained for FcγRIII were analysed by microdensitometry.

Double staining

Serial sections of each tissue were co-incubated in relevant pairs of the following antibodies: BRIC 128 at 14 μg/ml, 3G8 at 25 μg/ml, a mouse monoclonal IgG1 anti-human fibrillin-1 (11C1.3 clone; Neomarkers, Fremont, CA) at 40 μg/ml, a rabbit polyclonal anti-collagen VI antibody (gift of Dr C. Kielty, Manchester University, UK) at a dilution of 1:10, a mouse monoclonal IgG1 anti-collagen IV (Dako) at 10 μg/ml and a mouse monoclonal IgG1 anti-collagen laminin (Dako) at 12 μg/ml. Tissues were then incubated in a mixture of fluorescein- and rhodamine-conjugated goat antibodies to mouse IgM, mouse IgG1 and/or rabbit immunoglobulins (Sera-Lab, Crawley Down, UK), at final dilutions of 1:20, for 30 min. Tissues were washed and mounted in diamino bicyclo-octane. All double-stained sections were compared with single-stained and second-layer control sections.

Cell identification

In the absence of an available antibody with high specificity for FcγRIIIa versus FcγRIIIb, measures were taken to ensure discrimination of FcγRIII$^+$ cell populations. Comparison of counterstained and uncounterstained immunoperoxidase staining for FcγRIII, and immunoperoxidase staining for the macrophage marker CD68 indicated that the shape of the unstained nuclei of macrophages, granulocytes, or lymphocytes, with FcγRIII$^+$ cytoplasm, on uncounterstained sections, together with nuclear to cytoplasmic ratio allowed reliable discrimination of cell types in normal tissues except for the mononuclear cell types in densely packed lymphoid tissue and bone marrow.

Microdensitometry

Semiquantitative analysis of FcγRIII expression was performed on sections stained with the immunoperoxidase technique. As far as possible two specimens for each tissue were analysed microdensitometrically (Table 1). Sections were stained using supraoptimal concentrations of primary antibody and rigidly standardized incubation times. Analysis was performed using an M85 Vickers scanning microdensitometer (Vickers Medical Instruments, Sidcup, Kent, UK).[22] Absorption was measured at 550 nm with a mask diameter corresponding to a 5-μm section. Absorption was measured as relative extinction, in arbitrary units, using unstained areas of the section as reference. Measurements were taken over the cytoplasm of macrophages showing maximum staining in each of 10 randomly selected high-power fields. Mean readings and standard errors were obtained for each sample. Measurements from each staining batch were normalized by defining 1 unit as the value obtained for macrophages in a normal synovial intima included in each batch.

RESULTS

Cellular staining

Measurable levels of staining for FcγRIII were present on synovial intimal, and to a lesser degree subintimal, macrophages, alveolar macrophages, pericardial macrophages,

Table 1. Semiquantitative assessment of macrophage FcγRIII expression in normal tissues

Tissue	Macrophage population	FcγRIII Index*	Source
Synovium	Intimal macrophages	1	Biopsy
		1·28 ± 0·09*	Biopsy
		0·72 ± 0·04*	Biopsy
	Subintimal macrophages	0·38 ± 0·02	Biopsy
		0·35 ± 0·04	Biopsy
Lung	Alveolar macrophages	0·99 ± 0·10	Autopsy
		0·99 ± 0·08	Autopsy
Pericardium	Macrophages	1·03 ± 0·07	Biopsy
		0·92 ± 0·08	Biopsy
Liver	Kupffer cells	1·50 ± 0·09	Autopsy
		0·42 ± 0·04	Biopsy
Stressed dermis (elbow)	Macrophages	0·68 ± 0·07	Biopsy
Stressed dermis (toe)		0·27 ± 0·07	Biopsy
Salivary gland	Macrophages	0·43 ± 0·05	Biopsy
Placenta	Hofbauer cells	0·39 ± 0·04	Biopsy
		0·39 ± 0·03	Biopsy
Umbilical cord	Intravascular monocytes	0·86 ± 0·04	Biopsy
Bone marrow	Macrophages/precursors	Not measured†	Biopsy
Skeletal muscle	Macrophages	Not measured	Biopsy
Spleen/tonsil	Macrophage subsets	Not measured	Biopsy
Breast		Undetectable	Autopsy
		Undetectable	Biopsy
Brain	Microglia	Undetectable	Biopsy
		Undetectable	Biopsy
Thyroid	Macrophages	Undetectable	Autopsy
		Undetectable	Autopsy
Spinal disc/enthesis	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy
Intestine	Macrophages	Undetectable	Autopsy
		Undetectable	Biopsy
Myocardium	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy
Prostate	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy
Flexor forearm dermis	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy
Uterus	Macrophages	Undetectable	Biopsy
		Undetectable	Autopsy
Kidney	Macrophages/	Undetectable	Autopsy
	Mesangial cells	Undetectable	Autopsy
Gallbladder	Macrophages	Undetectable	Autopsy
Nailbed	Macrophages	Undetectable	Biopsy
Unstressed dermis	Macrophages	Undetectable	Biopsy
		Undetectable	Biopsy

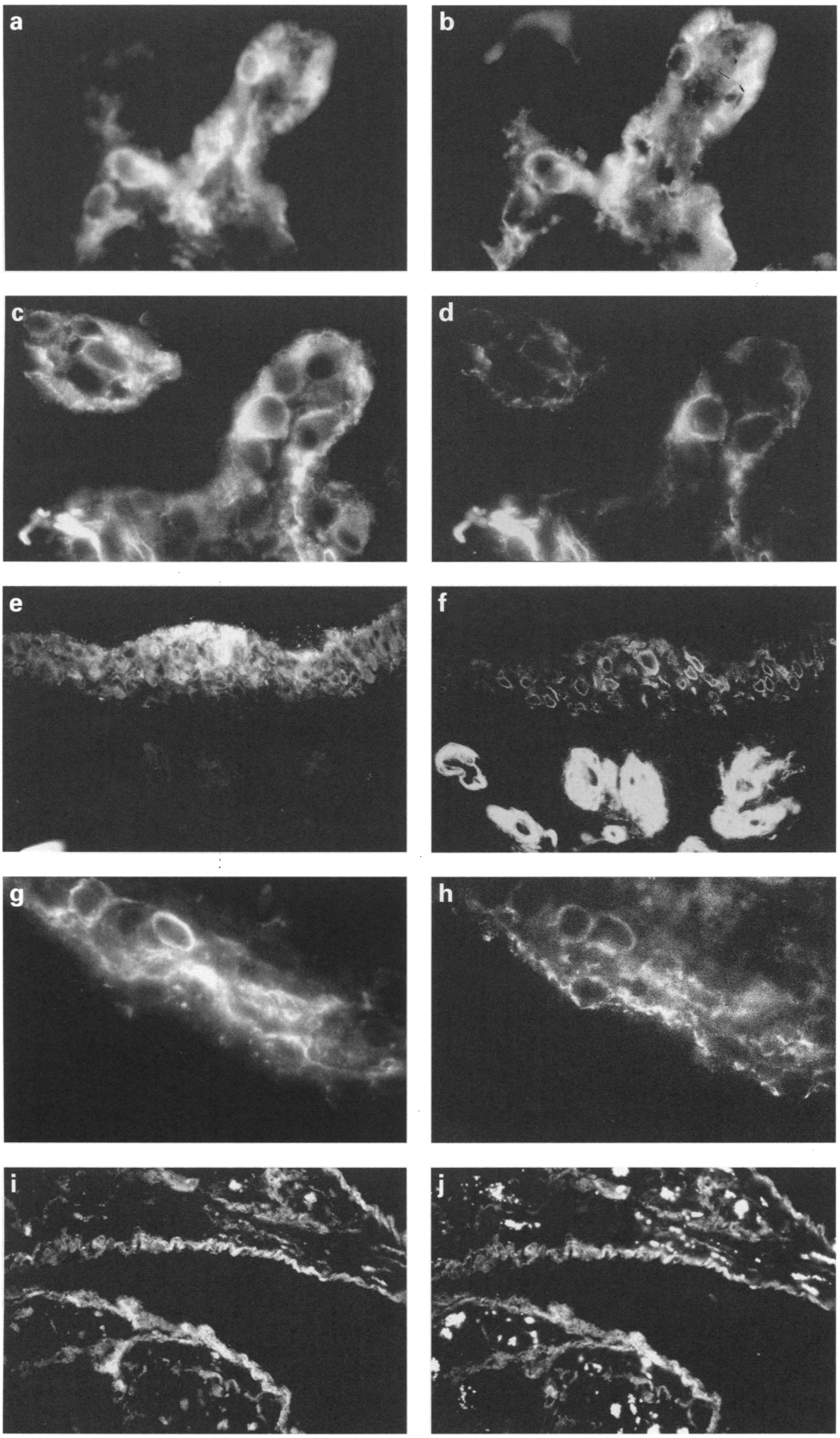
*Mean relative extinction for immunoperoxidase staining for FcγRIII normalized to synovial intima = 1, ± standard error. Comparative values for two synovial samples given in parentheses.

†See text, index not derived for technical reasons.

Kupffer cells, macrophages in mechanically stressed dermis, salivary gland macrophages, placental Hofbauer cells (macrophages), and monocytes in umbilical cord vessels (Table 1). Macrophage FcγRIII staining was also observed in muscle, bone marrow and lymphoid tissue, but was not measured for technical reasons. In other tissues, FcγRIII staining was not seen on macrophages despite their presence, as indicated by CD68 staining.

In muscle very few FcγRIII$^+$ cells were found in comparison to the number of CD68$^+$ cells present. The level of FcγRIII staining on this minority of macrophages was significant, but the etiolated profile of the cells precluded reliable measurement using the standard mask. Large FcγRIII$^+$ mononuclear cells consistent with macrophages were seen in bone marrow and some samples of tonsil and splenic white pulp. However, it was not possible to be certain of the identity and state of maturation of these cells. Moreover, in other samples of tonsil virtually no staining was seen other than on scattered granulocytes.

Staining with antibodies to FcγRI and FcγRII confirmed the specificity of differential staining obtained for FcγRIII in synovium. As reported in diseased tissue,[23] all macrophages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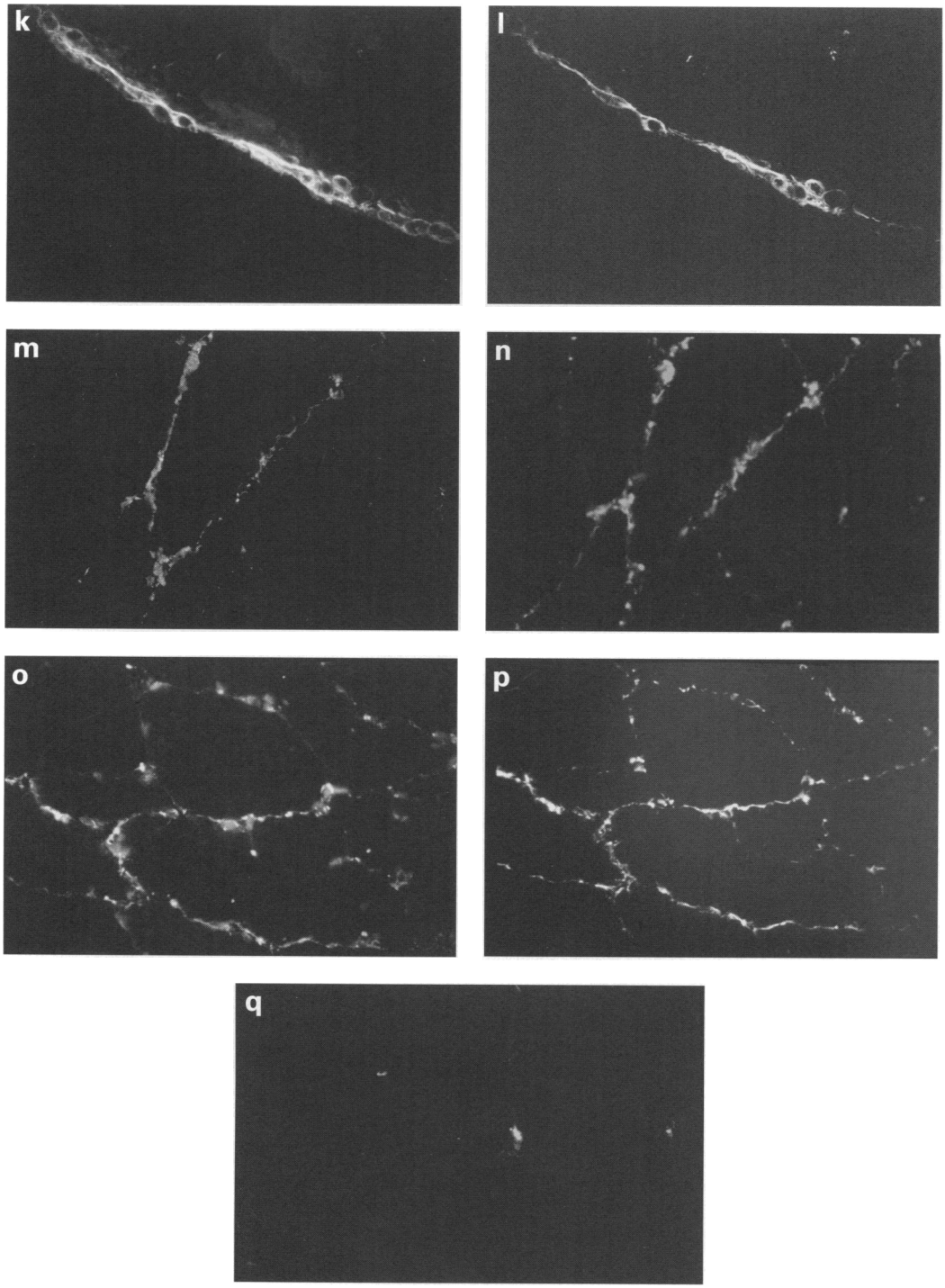
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stained for FcγRII and FcγRI staining was limited to subintima (not shown).

FcγRIII$^+$ granulocytes were seen sparsely scattered in most tissues and in large numbers in splenic white pulp. Comparison of both granulocyte and macrophage staining in post-mortem and biopsy samples indicated that the source of tissue had no significant effect on staining levels.

Extracellular staining

Extracellular staining for FcγRIII was restricted to synovial intima, mesothelia, skeletal and cardiac perimysium, uterine arterial internal elastic lamina and dermis at sites of mechanical stress. In synovial intima FcγRIII staining was chiefly cellular and pericellular, and less often on isolated fibrillar structures. Pericellular FcγRIII and DAF (Fig. 1a,b) frequently co-localized around the same cells in a pattern which matched closely to that of fibrillin-1 (Fig. 1c,d). Extracellular DAF staining was not confined to sites of fibrillin-1 but showed some apparent overlap with pericellular staining for laminin (Fig. 1e,f). Neither DAF nor FcγRIII staining co-localized with collagen VI-containing fibrillar structures (Fig. 1g,h) or with collagen IV, which did not show a pericellular or fibrillar pattern (not shown).

In pericardium, and on the peritoneal surface of spleen, linear staining for both FcγRIII and DAF was observed (Fig. 1i,j). For pericardium, FcγRIII and DAF staining was discontinuous with both brightly stained and unstained areas (Fig. 1k). Linear staining co-localized with fibrillin-1 (Fig. 1k,l) and not collagen VI (not shown). A similar pattern of staining was seen on scattered fibrous septae between cardiac myocytes (Fig. 1i,j).

Linear staining for both FcγRIII and DAF was observed on skeletal muscle epimysium (Fig. 1m,n). Staining patterns for the two molecules demonstrated major overlap and followed the pattern of fibrillin-1-based microfibrils, which formed a discontinuous perimyseal network (Fig. 1o,p). Collagen-VI-based microfibrils formed a continuous perimyseal envelope (not shown), which, although closely apposed to fibrillin-1-based microfibrils, did not match FcγRIII and DAF staining. CD68 staining indicated that only a fraction of epimysial staining for FcγRIII and DAF could be accounted for by macrophage cytoplasm/membranes (Fig. 1q).

A proportion of small to medium-sized uterine arteries showed FcγRIII staining of the internal elastic lamina, but vessels in other tissues did not stain.

Extracellular matrix-associated FcγRIII was assumed to represent FcγRIIIa, being associated with FcγRIII$^+$ macrophages and not granulocytes. In muscle, although FcγRIII$^+$ macrophages were scarce, CD68 staining occasionally revealed macrophages with a cytoplasmic extension of similar extent to a stretch of microfibril-associated FcγRIII staining suggesting that FcγRIIIa may be deposited by transient macrophage filopodia. In one sample of dermis exposed to mechanical stress granulocytes were present, raising the possibility that fibrillar staining was due to FcγRIIIb in this case.

In keeping with previous reports,[7] extracellular DAF staining was observed in many tissues, but with major variation in intensity. A proportion of DAF staining precisely co-localized with a subset of fibrillin-1-containing fibrillar elements, including large elastic fibres in tissue stroma but not vascular internal elastic lamina. DAF was also present in epithelial basal laminae. DAF did not co-localize with collagenVI.

DISCUSSION

The findings presented indicate that macrophage FcγRIIIa expression varies widely between different tissues. FcγRIIIa expression by monocytes following 'maturation' *in vitro*[24] appears to mirror macrophage behaviour in synovial intima, hepatic sinusoids and alveoli, but not, for instance, gut, skin, or brain. Low-level expression of FcγRIIIa by macrophages in these latter tissues is not ruled out and might be detectable with more sensitive methods, but the differential in FcγRIIIa expression between tissues demonstrated is likely to be of major functional significance.

Although levels of staining for FcγRIII were reproducible in most tissues (Table 1), lymphoid tissues and liver showed major variation. This suggests that for these tissues FcγRIII expression may vary with immune reactivity or as part of an acute phase response.

FcγRIIIa expression has been found to be induced by

Figure 1. Immunofluorescent staining of normal human tissues for FcγRIII, DAF and matrix components. (a) and (b) Synovial intimal villus stained for FcγRIII and DAF showing unexpected co-localization of pericellular staining; final magnification ×400. (c) and (d) Synovial intimal villus stained for DAF and fibrillin-1, both pericellular (middle left) and fibrillar (top right) staining patterns co-localize; final magnification ×400. (e) and (f) Synovium stained for DAF and laminin (intima at top, subintimal vessels below). Co-localization of pericellular staining is less consistent than for DAF and fibrillin-1. Even where staining for DAF and laminin outlines the same cell, the pattern appears concentric rather than identical; final magnification ×200. (g) and (h) Synovial intima stained for DAF and collagen VI. The images are totally discordant, with the exception of one elliptical cell outline (top centre). On precise registration the outlines of this cell are partially concentric, and not coincident; final magnification ×400. (i) and (j) Pericardium stained for FcγRIII and DAF showing an area of bright continuous curvilinear staining of the mesothelium and some associated fibrous septa. Macrophages are sparse at this site and account for only a small fraction of FcγRIII staining. Autofluorescent glycogen granules are also present in the underlying tissue, seen more brightly on the rhodamine channel (j); final magnification ×200. (k) and (l) Pericardium stained for DAF and fibrillin-1. Pericellular and extracellular linear staining for DAF co-localizes almost entirely with fibrillin-1. This area of bright staining merges with an area of minimal staining at bottom left; final magnification ×200. (m) and (n) Muscle stained for FcγRIII and DAF. The images share many common elements but are not identical; final magnification ×200. (o) and (p) Muscle stained for DAF and fibrillin-1. The images show close correspondence, on fibrillar structures outlining individual muscle cells; final magnification ×200. (q) Muscle stained for CD68. This field is of a serial section of the field shown in (o) and (p), demonstrating that the FcγRIII staining in (o) is not accounted for by cellular staining of macrophages; final magnification ×200.

transforming growth factor-β (TGF-β) and interleukin-10 and down-regulated by interleukin-4.[25,26] It is likely that the relative availability of these cytokines in liver, lung and synovium favours FcγRIIIa expression. The appearance of FcγRIIIa on synovial intima at the time of embryonic joint cavity formation[6] and in mechanically stressed dermis suggests that at these sites TGF-β may be produced locally in response to mechanical stimuli.[27] Moreover, fibrillin-1-based microfibrils are known to bind a wide range of immunologically active ligands, including the latent TGF-β-binding proteins.[28–32] Fibrillin-1-based microfibrils may therefore provide a reservoir of latent TGF-β.

The techniques used in this study permit the conclusion that both extracellular FcγRIII and DAF associate predominantly with fibrillin-1-based structures and it is tempting to suggest a direct interaction with fibrillin-1 itself. However, such an interaction will require demonstration by further analytical and ultrastructural studies.

The role of FcγRIIIa$^+$ macrophages may vary from tissue to tissue. Clearance of small immune complexes by liver and spleen provides a means of preventing dissemination of toxins via the circulation. Alveolar macrophages are at an interface with the environment where recognition of small amounts of antigen may be important. The role of FcγRIIIa in synovium and mesothelia is less clear. However, the close association of FcγRIIIa with DAF and fibrillin-1 in these tissues suggests some co-ordinated function in the protection of internal body interfaces. These tissues are unlikely to encounter foreign antigen often, but a rapid response to antigen may help prevent the development of sequestered infection within a cavity. Anecdotal accounts of intra-articular antigen administration suggest that it is a potent route for immunization (R. R. Coombs; personal communication).

Synovium is a major target for autoimmune disease, particularly in the form of rheumatoid arthritis (RA). Moreover, the pattern of FcγRIIIa expression observed fits closely with the pattern of extra-articular involvement in RA. FcγRIIIa signals via two common FcR γ-chains,[33] which in macrophages gives rise to production of mediators such as tumour necrosis factor-α (TNF-α). In RA subjects, events occurring in synovium, liver, lung, pericardium and bone marrow are consistent with the local production of TNF-α by macrophages in response to FcγRIIIa ligation by small immune complexes, such as IgG rheumatoid factor dimers, found in the circulation in RA.[34]

The significance of microfibril-associated FcγRIII, particularly in muscle, is puzzling. Binding to extracellular FcγRIII should not lead to macrophage cytokine production, but may allow aggregation of small complexes. This may lead, in the presence of DAF on fibrils and CD59 on muscle cells, to controlled generation of C3a. Perhaps perimysium acts as an extravascular sump for complexes in the way that red cells, through complement receptor 1,[35] do for circulating complexes. Binding of complexes in muscle may contribute to viral myalgia and may also be of relevance to muscle pain and stiffness in polymyalgia rheumatica (PMR). Circulating immune complexes occur in PMR, but differ from those found in RA in their content of alternative pathway components.[36] Muscle biopsies show IgG and fibrin deposits in the perimysium.[37] RA and PMR show major overlap, but most cases fall into one or other category. This suggests that whether the impact of extravascular complexes is predominantly at sites of cell-associated or matrix-associated FcγRIIIa depends on detailed characteristics of the complexes such as size, interactions with complement pathways (as in PMR) and local synthesis of both antigen and antibody (as for IgG rheumatoid factors in RA synovium).[6,38]

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