

ICAM-1 expression on endothelium and systemic cytokine production in cutaneous neutrophilic leukocytoclastic vasculitis in NZBxNZWF₁ mice

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Summary. The present study has examined relationship between cutaneous microvessel injury and adhesion molecule expression on the endothelium by cytokines in NZBxNZWF₁ (B/WF₁) mice, a model for human systemic lupus erythematosus. In advanced ages associated with overt clinical manifestation, but not in early ages, neutrophils with a minor proportion of monocytes and lymphocytes mainly adhered to the endothelium of capillary and the venule with fragmentation (leukocytoclasia), leading to the vascular injury (leukocytoclastic vasculitis). This was confirmed by the leak of monstral blue from the blood vessel. At this stage LFA-1⁺ leukocytes adhered to intensely expressed ICAM-1 on the endothelium, and this was paralleled with a significant rise in IL-1 α and TNF- α in the circulation. The present study suggests that IL-1 α and TNF- α may, at least in part, be responsible for the increased ICAM-1 expression on endothelium in cutaneous microvessels, resulting in the vascular injury characterized by neutrophilic leukocytoclasia in B/WF₁ mice.

Key words: B/WF₁ mouse, Microvessel, Adhesion molecule, Neutrophil, Cytokines, Vasculitis, Endothelium, Leukocytoclasia

Introduction

As NZBxNZWF₁ (B/WF₁) mice, an animal model for autoimmune human systemic lupus erythematosus (SLE), grow older, they produce especial autoantibodies against nuclear antigens, double-stranded DNA (Lambert and Dixon, 1968; Tokado et al., 1991) and gp70, a major envelope glycoprotein of endogenous retrovirus (Izui et al., 1979). Then, immune complexes (ICs) are formed in the circulation, resulting in the deposition not only in glomeruli but also in widespread

blood vessels such as skin, lungs, salivary glands and other organs (Crowson et al., 2003).

There are several types of vascular lesions in different types and sizes of blood vessels (e.g., fibrinoid degeneration, mononuclear-cell perivascularitis, polyarteritis nodosa-like vasculitis, or leukocytoclastic vasculitis) in patients with SLE or in lupus model mice (Funata, 1979; Moyer et al., 1987; Mathieson et al., 1993; Belmont et al., 1996; Pique et al., 2002). There is general agreement that arthus type-III reactions occur when ICs activate the direct complement cascade sequence and that vasoactive amines (e.g., histamine and serotonin from basophils and platelet degranulation) (Camussi et al., 1981; Medcalf et al., 1982) play a role in vascular damage. However, expression of adhesion molecules on endothelium by cytokines in microvessel (e.g., arteriole, venule and capillary) of cutaneous tissues, and the adhesion of leukocytes to their endothelium is not well understood (Henninger et al., 1997).

Adhesion molecules are involved in firm adhesion of circulating leukocytes to endothelial cells. Among several cytokines intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 especially, which are members of the Ig superfamily, are expressed on activated endothelial cells (Springer, 1995), and ICAM-1 acts on β 2 integrin (e.g., lymphocyte function-associated antigen; LFA-1 and Mac-1) on all types of leukocytes, whereas VCAM-1 acts on α 4 integrin (e.g., very late activation antigen; VLA-4 and lymphocyte Peyer's patch HEV adhesion molecule; LPAM-1) on lymphocytes and monocytes respectively (Alon et al., 1995). Enhanced ICAM-1 and/or VCAM-1 expression on endothelial cells has been demonstrated in MRL/lpr or B/WF₁ mice, and among several cytokines interleukin (IL)-1 α , IL-1 β and tumor necrosis factor(TNF)- α sequentially induce endothelial ICAM-1 and VCAM-1 expression in these autoimmune strains of mice (Boswell et al., 1988a,b). Furthermore, disease progress in MRL/lpr mice can be attenuated by anti-ICAM-1 mAb or by back-crossing with ICAM-1 gene-targeted mice (Bullard et al., 1997).

In addition ICAM-1 plays a major role in the development of glomerular injuries in patients with active SLE (Belmont et al., 1994; Bullard et al., 1997), and LFA-1-expressing leukocytes interacting with ICAM-1 expression on glomerular endothelium caused glomerular damage, at least in part, during the active phase in B/WF₁ mice (Kameyama and Hayashi, 1994).

In this study we examined the cutaneous microvascular pathology in relation with cytokine production (IL-1 α and TNF- α) in the circulation and expression of adhesion molecules (ICAM-1 and VCAM-1) on the endothelium in B/WF₁ mice.

Materials and methods

Mice

Two-week-old female-specific pathogen-free NZB \times NZWF₁ (B/WF₁) mice (n=38 totally) were obtained from SLC Japan Co. (Shizuoka, Japan). The animal experiments were approved by the Animal Research Ethics Board of Faculty of Agriculture, Yamaguchi University.

Sampling

During the experimental periods (2 to 8.25 months), plasma and urine were obtained at the times indicated. At the age of 5, 6 and 8.25 months, mice were killed by euthanasia, and the blood for cytokine assays and back skin for histopathology (left half) and immunohistochemistry (right half), were sampled. Macroscopy of skins revealed slight erythematous and edematous changes at the age of 8.25 months. The number of mice used in each assay was described below.

IgG2a anti-nuclear antibody (ANA)

ANA titer in the blood (plasma or serum; n=9, 5, 8 or 11 at the age of 2, 5, 6 or 8.25 months respectively) was determined by indirect immunofluorescence by the method described previously (Hasegawa and Hayashi, 2003). In brief, frozen liver sections from four-week-old female BALB/c mice (SLC Co., Shizuoka, Japan) were incubated with a serial two-fold-diluted sample and then incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG2a (BETHYL, Montgomery, TX, USA), which is a pathogenic Ig subclass for deposition in glomeruli (Hasegawa et al., 2002). The titer, expressed as the reciprocal of the highest dilution of sample showing positive nuclear fluorescence, was transformed to log₂.

Clinical findings

Urine protein and urine leukocyte sediment (n=10, 16 or 10 at the age of 2.5, were assessed semiquantitatively using dip sticks (Multi-sticks SG-L, Bayer, Tokyo, Japan). The amounts of creatinine in

blood (n=10, 8, 8, 17 or 17 at the age of 2.5, 5, 6, 7 or 8.25 respectively) were determined using Fuji dry-chem 5500S and Fuji dry-chem slides (Fuji film Co., Tokyo, Japan).

Value of IL-1 α and TNF- α in blood by ELISA

The concentration of IL-1 α and TNF- α (n=9, 8 or 11 at the age of 5, 6 or 8.25 months respectively) in each blood sample was measured by mouse ELISA kits (IL-1 α ; Genzyme Techn., Minneapolis, MN, USA and TNF- α ; Cosmo Bio Co., Tokyo, Japan). The minimal detectable concentration was 2.5 pg/ml for IL-1 α and 3 pg/ml for TNF- α .

Immunohistochemistry

Frozen sections from cutaneous tissues (n=3, 2 or 3 at the age of 5, 6 or 8.25 months respectively) were made and fixed in acetone as described previously (Hasegawa and Hayashi, 2003). Each section was incubated with either FITC-labelled goat anti-mouse IgG2a or FITC-labelled goat anti-mouse C3 antibody (Cappel, Durham, NC, USA).

Sections were also incubated with rat monoclonal antibody (mAb) against ICAM-1 (clone KBA; Sekagaku, Tokyo, Japan), VCAM-1 (Antigenix, NY, USA), LFA-1 (clone KBA; Sekagaku, Tokyo, Japan), or VLA-4 (Antigenix, NY, USA), and rabbit anti-mouse platelet (Inter-cell technologies, Hopewell, NJ, USA), and then sections were incubated with peroxidase-conjugated goat anti-rat IgG antibody (ICN Pharmaceuticals, Ohio, USA) or FITC-conjugated goat anti-rabbit IgG (COOPER Biomedical technol., Nalvern, PA, USA). For immunoperoxidase, sections were then reacted with 3,3'-diaminobenzidine tetrahydrochloride dihydrate in Tris-HCl buffer (pH 7.6) containing H₂O₂, and counterstained with Hematoxylin.

As a negative control for direct immunofluorescence assay, cutaneous tissues from four-week-old female BALB/c mice were used. For indirect immunofluorescence or immunoperoxidase assays, the reaction without primary antibody served as a negative control and kidneys from female NZB \times NZWF₁ mice with overt disease at the age 8 months were used as a positive control.

Histopathology, and evaluation of adherence of neutrophils to endothelium and ICAM-1 expression on endothelium in microvessel

Cutaneous tissues were fixed in 10% neutral-buffered formalin (pH 7.4) and embedded in paraffin, and sections (4 μ m) were stained with hematoxylin and eosin (HE) and toluidine blue.

The degree of attachment of leukocytes to endothelial cells (index of neutrophil attachment to endothelium: I.N.A.E.) in capillary (n=9, 8 or 11 at the age of 5, 6 or 8.25 respectively) was estimated

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semiquantitatively with a 0-2 scale as follows; no leukocyte (0), the presence of neutrophils in the lumen (1), and attachment of neutrophils to the endothelium (2). The expression of ICAM-1 on the endothelium (I.I.E.) was evaluated semiquantitatively by the method with minor modification described previously (Kameyama and Hayashi, 1994) based on intensity on a 0-3 scale with faint (0), slight (1), moderate (2) or marked (3).

A total 10-20 microvessels were calculated using the following formula. I.N.A.E. or I.I.E. = $(n_0 \times 0) + (n_1 \times 1) + (n_2 \times 2) + (n_3 \times 3) / \Sigma n$. Ten-twenty microvessels of each skin tissue were examined blindly by two different observers.

Evaluation of vascular damage

Mice at the age of 8.5 (n=4) months were injected intravenously (tail vein) at a 2-hour interval (total 3 times) with 0.5 ml monstral blue B (copper phthalocyanine; Sigma, St. Louis, MO, USA) / mouse, which is colloidal dye having 50 nm diameter and used as a parameter of increased permeability (Joris et al., 1982), and the central back skin was obtained one hour after the last injection and processed for histopathology and/or immunohistochemistry.

Statistical analysis

The data are expressed as the mean of examined samples \pm standard error (SE), and are analyzed by the one-way, unpaired Student's t-test to evaluate the significance of differences. The Pearson's correlation coefficient was used to assess correlation between the I.I.E. score and production of IL-1 α and TNF- α or the I.N.A.E. score; a P value less than 0.05 was considered significant.

Results

IgG2a ANA titer

As shown in Fig. 1A, there was little or no detectable ANA at the age of 2 months, but then the ANA titer began to develop at the age of 5 months. Its titer increased until 8.25 months of age ($P < 0.01$; compared with that in mice at the age of 2 months).

Protein and leukocyte in urine, and creatinine in blood

High protein (Fig. 1B; $P < 0.01$; compared with those in mice at the age of 2.5 months) and leukocyte (Fig. 1C) values in urine were found at the age of 7.75 and 8 months respectively. A high creatinine (Fig. 1D) value was observed at the age of 8.25 months.

Cytokines in circulation by ELISA, and I.N.A.E.

A low concentration of IL-1 α was already detected

at the age of 5 and 6 months, and it increased until 8.25 months ($P < 0.001$; compared with that in mice at the age of 5 months (Fig. 2A). On the other hand, an increased concentration of TNF- α was detected at the age of 8.25 months (Fig. 2B; $P < 0.001$; compared with that in mice at the age of 5 months).

I.N.A.E. (the degree of attachment of neutrophils to endothelial cells) began to increase at the age of 6 months (Fig. 2C; $P < 0.01$; compared with mice at the age of 5 months). At the age of 5 months a few neutrophils were present and they increased within the blood vessel at the age of 6 months. Adherence of neutrophils to the endothelium increased at the age of 8.25 months.

I.I.E. (the intensity of ICAM-1 expression on the endothelium) lineally increased in aging and the score at the age of 8.25 months was significantly higher than that at the age of 5 month (Fig. 2D; $P < 0.01$). I.I.E. score correlated positively and statistically with the levels of serum IL-1 α (Fig. 3A; $P < 0.05$) and TNF- α (Fig. 3B; $P < 0.01$) or I.N.A.E. score (Fig. 3C; $P < 0.05$).

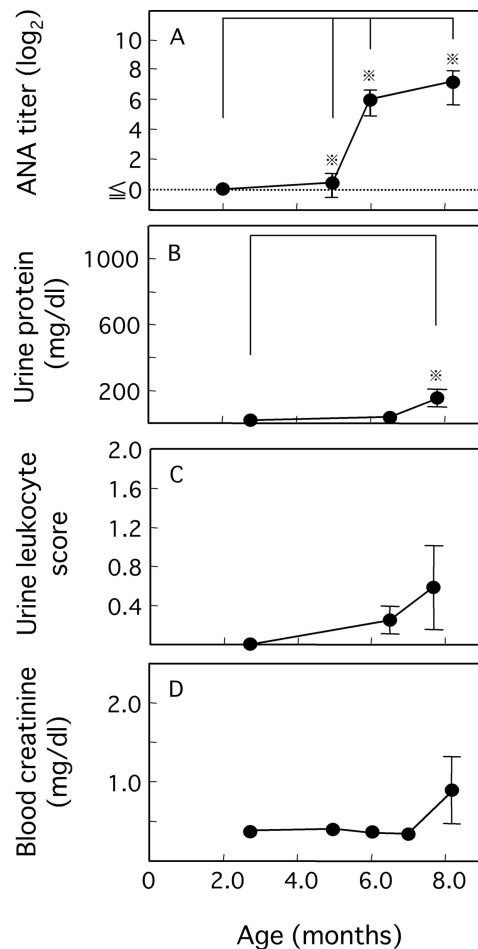


Fig. 1. ANA (A) is detected at the age of 5 months, and its titer increases in aging. Age-related increased excretion of protein (B) and leukocytes in urine (C), and creatinine (D) in blood are also observed. Each point represents the mean \pm SE from 5-17 mice. *: $P < 0.001$

Detection of platelets, and deposits of IgG2a and C3 in microvessel

Platelets, with or without aggregation in the lumen, or their adherence to the endothelium, and granular deposits of IgG2a and C3 on the endothelium and basement membrane were already seen at the age of 5 and 6 months, though in general their degree was weak and variable. An increase in intensity was observed at the age of 8.25 months (Fig. 4A, B).

Expression of ICAM-1, VCAM-1, LFA-1 and VLA-4

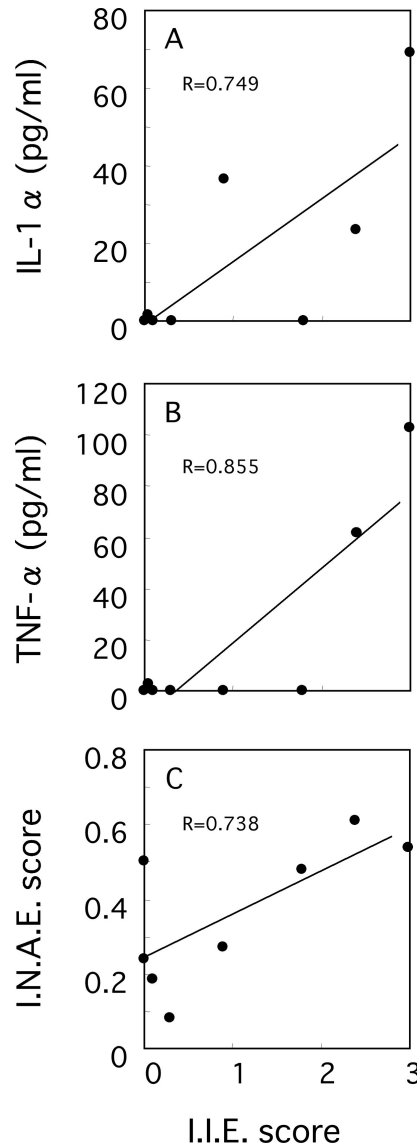
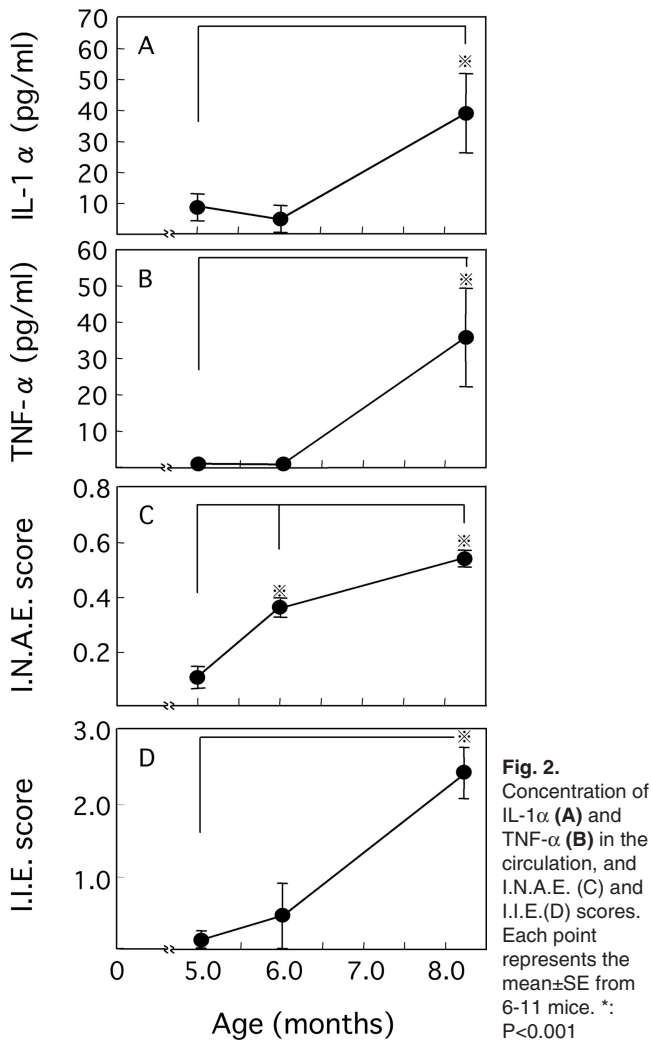
In general, the expression of ICAM-1 (Fig. 5A) and VCAM-1 on the endothelium was faint at the age of 5 months, and its expression increased intensely at the age of 8.25 months (Fig. 5B), although the degree of adhesion molecule expression varied in each individual. In the endothelium VCAM-1 expression was paralleled

with ICAM-1 expression, although the former was less prominent than the latter. Leukocytes in and around blood vessels or attached to endothelial cells expressed not only ICAM-1 but also LFA-1. VLA-4-positive leukocytes in blood vessels were few.

Leak of monstral blue was observed in affected microvessels (Fig. 5C).

Histopathology of microvessel in subcutaneous tissue

In general, microvessel lesions were observed mainly in capillary and venule and deep subcutaneous tissues, and they were less severe at the age of 5 (Fig. 6A) and 6 months (Fig. 6B). These lesions increased at the age of 8.25 months (Figure 6C-E). At this stage an



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increased number of neutrophils with some monocytes and lymphocytes within the vascular lumen and attachment of leukocytes to endothelium were observed. Also, leukocytes infiltrated around or were close to microvessels in the interstitial tissues. No basophils were observed in the microvessel lumen. In affected vessels,

there was swelling, proliferation, degeneration and desquamation of endothelial cells (but not in all the endothelium of vessels in an individual animal). At these sites, nuclear fragmentation of neutrophils (leukocyte clasis) with destructed endothelium and vascular walls was also observed. Some such vessels were accompanied with hemorrhagic changes, and had microthrombosis. Leuko-occlusive vasculopathy by neutrophils was sometimes observed. Fibrin deposition in the microvessels was only minimal. Hyperemia and edema of dermis and subcutaneous tissues were constantly observed. Collagen necrosis of interstitium and fibrinoid necrosis in arterioles were rarely observed. There was a difference in the number of mast cells neighbouring vessels at the age of 8.25 months compared with those at younger ages (5 months). Mast cells with or without degranulation were enlarged and increased slightly, and neutrophils were scattered in the cutaneous tissues in aging. Mononuclear cell-

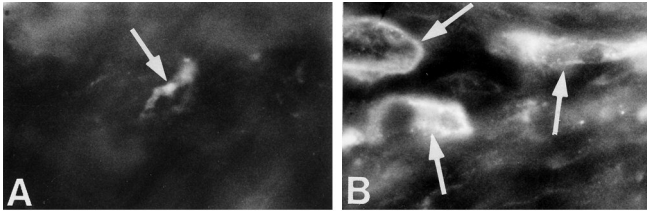


Fig. 4. Immunohistochemistry of the presence of platelets (A, an arrow) and heavy deposits of C3 (B, arrows) in microvessels at the age of 8.5 months. Immunofluorescence. x 200

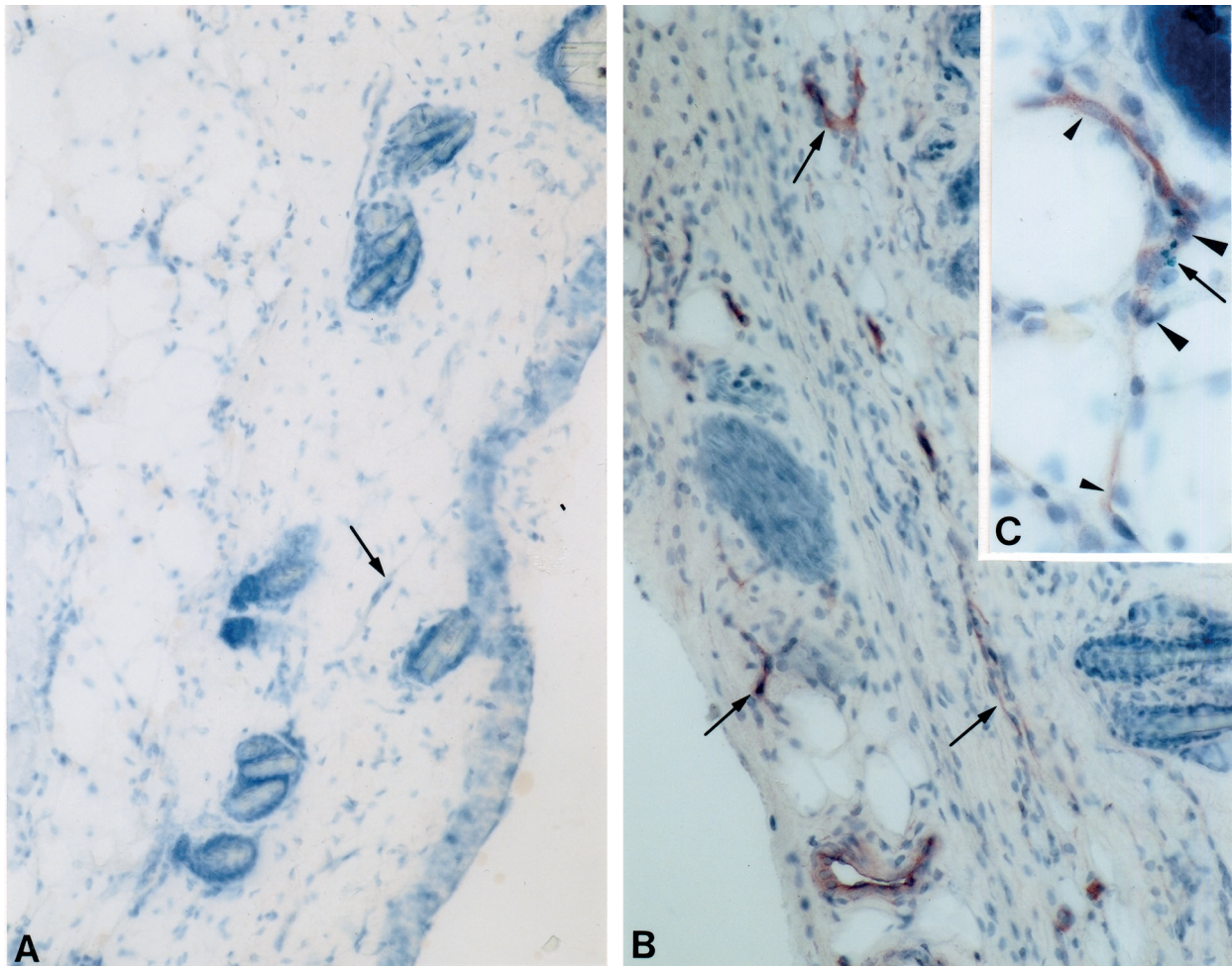


Fig. 5. Compared to faint ICAM-1 expression at the age of 5 months (A, an arrow), its expression on endothelial cells in microvessels increased at the age of 8.25 months (B, arrows). Leak of monstral blue (C, an arrow), endothelial cells (small arrow heads) and neutrophils (large arrow heads) are visible (C). Immunoperoxidase. A, B, x 200, C, x 400

perivasculitis was also rarely observed.

Discussion

The present study has demonstrated cutaneous leukocytoclastic vasculitis, especially in capillaries and venules (Sanchez-Perez et al., 1993, 1996; Pique et al., 2002) which results in vascular destruction. This was confirmed by monstral blue leak from the blood vessels (Joris et al., 1982). LFA1⁺ leukocytes consisting mainly of neutrophils reacted with intensely-expressed ICAM-1 on endothelial cells. The increased expression of ICAM-1 was paralleled with the increased systemic production of IL-1 α and TNF- α in the circulation in B/WF1 mice with overt disease, suggesting that the expression of ICAM-1 may be induced synergistically by those cytokines in lupus model mice (Wuthrich et al., 1990; Wuthrich, 1992; Henninger et al., 1997) and in active human SLE (Maury and Teppo, 1989; Gabay et al., 1997). Moreover, the leukocytic reaction pattern in aging in cutaneous capillary vessels here coincided with that in glomerular vessel in B/WF₁ mice (Kameyama and Hayashi, 1994). Furthermore, although the pattern of VCAM-1 expression on the endothelium was similar to that of ICAM-1, their roles in vascular injury may be minor, since VLA-4⁺ mononuclear cells in the blood vessel were few. In addition, anti-DNA autoantibody (Lai et al., 1996) and CpG oligodeoxynucleotides in ICs (Miyata et al., 2001) can also induce expression of those adhesion molecules on endothelial cells other than

cytokines. Thus, it seems likely that the mechanisms of adhesion molecule expression might be complicated.

The origin of the increased circulating these cytokines may be derived from mononuclear cells (macrophages and lymphocytes) in lympho-reticular organs such as lymph nodes (Boswell et al., 1988a,b; Prud'Homme et al., 1995; Sun et al., 2000). Circulating those cytokines may also further stimulate IL-1 α , IL-1 β and TNF- α production from endothelial cells (Henninger et al., 1997; Vadeboncoeur et al., 2003), which may act by autocrine mechanisms (Crowson et al., 2003) and play a role in induction of adhesion molecule expression.

Vascular destruction may be mediated by oxygen products (Niwa et al., 1985; Suwannaroji et al., 2001) and release of lysosomal enzymes (Belmont et al., 1996) by neutrophils attached to the endothelium via adhesion molecules discussed above at the site of ICs deposition (Suzuki et al., 2003). Circulating ICs leading to endothelial cell injury with activation of the clotting pathway (Crowson et al., 2003) may be minor, since microthrombosis in microvessels was rarely seen. It has also been reported that antibodies directed to endothelial antigenic targets (Crowson et al., 2003) or autoantibodies against myeloperoxidase in cytoplasm of neutrophils, might evoke vasculitis (Falk and Jenette, 1988; Mathieson et al., 1993; Sen and Isenberg, 2003). In addition mast cells neighboring microvessels may play some roles in vascular injury (Norman et al., 2003; Yanabe et al., 2003). Further study is needed to clarify

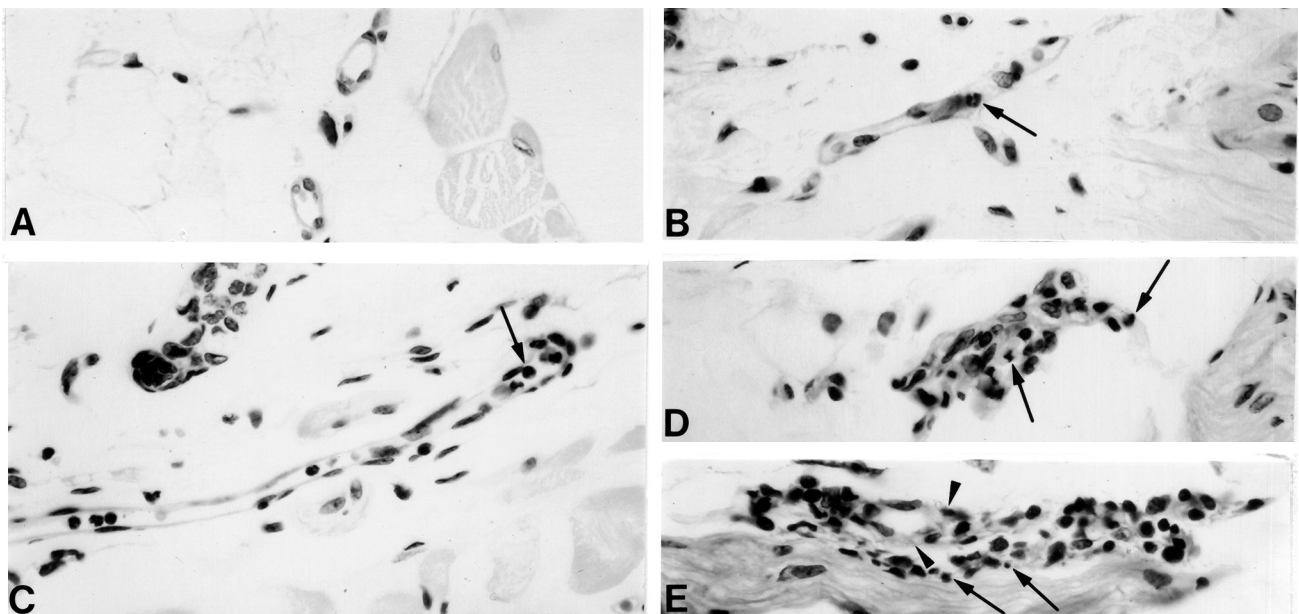


Fig. 6. Histopathology of microvessels in the subcutaneous tissues. Slightly enlarged endothelial cells without neutrophils in the lumen at the age of 5 months (A). Neutrophils in the lumen (B; an arrow), their attachment to endothelial cells (C; an arrow), destroyed neutrophils (D; arrows) in the lumen and walls, and leukocytoclastic neutrophils with fragmentation outside blood vessels (E; arrows) and destroyed vascular walls (arrow heads) are seen at the age of 8.25 months. HE. x 400

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the mechanisms of the fragmentation of neutrophils and the role of mast cells in vascular injury.

In conclusion, leukocytoclastic vasculitis in cutaneous microvessels of B/WF₁ mice with aging may, at least in part, be related to the interaction between ICAM-1 expression on the endothelium by the systemic production of IL-1 α and/or TNF- α and LFA-1⁺ neutrophils.

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