Important role for macrophages in induction of crescentic anti-GBM glomerulonephritis in WKY rats

Masato Isome1,2, Hidehiko Fujinaka1,3, Laxman P. Adhikary1, Pavel Kovalenko1, Adel G. Ahmed El-Shemi1, Yutaka Yoshida1, Eishin Yaoita1, Toshiyuki Takeishi4, Motohiro Takeya5, Makoto Naito4, Hitoshi Suzuki2 and Tadashi Yamamoto1

1Department of Structural Pathology, Institute of Nephrology and 4Second Department of Pathology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, 2Department of Pediatrics, Fukushima Medical University School of Medicine, Fukushima, 3Department of Pediatrics, Niigata National Hospital, Kashiwazaki and 5Department of Cell Pathology, Kumamoto University Faculty of Medical and Pharmaceutical Sciences, Kumamoto, Japan

Abstract

Background. A crucial role for CD8+ cells in induction of crescentic anti-glomerular basement membrane (GBM) glomerulonephritis (GN) in WKY rats was demonstrated in studies showing that depletion of CD8+ cells completely suppressed glomerular accumulation of monocytes/macrophages (Mo/Mφ), crescent formation and proteinuria. Because these studies did not definitively identify CD8+ cells as the cause of tissue injury, we examined the roles of Mo/Mφ in the development of anti-GBM GN.

Methods. We examined correlations between the amount of urinary protein and the numbers of glomerular CD8+ cells or Mo/Mφ in rats after administrating different doses of anti-GBM antibody (5.0, 7.5, 10.0 and 25.0 μl/100 g body weight). The roles of Mo/Mφ in induction of GN were examined in animals by depleting Mo/Mφ in the glomerulus. To do this, rats were injected intravenously with liposome-encapsulated dichloromethylene diphosphonate (liposome-MDP) from day 3 to day 7 after anti-GBM antibody injection and they were then sacrificed at day 8.

Results. Liposome-MDP treatment significantly reduced the number of ED-1+ Mo/Mφ accumulated in glomeruli from 32.1±1.2 to 1.4±0.3/glomerular cross-section (mean±SD, P<0.01), and the amount of urinary protein from 103.8±19.8 to 31.8±15.9 mg/day (P<0.01), as well as the incidence of crescentic glomeruli from 91.3±2.7 to 23.3±7.6% (P<0.01) at day 8. This treatment also reduced the number of CD8+ cells accumulating in the glomeruli from 5.4±0.7 to 0.5±0.1/glomerular cross-section (P<0.01). Upregulation of glomerular intercellular adhesion molecule 1 (ICAM-1) and monocyte chemoattractant protein 1 (MCP-1) mRNA expression was suppressed by Mo/Mφ depletion.

Conclusion. These results indicate that Mo/Mφ play an important role in the induction of crescentic anti-GBM GN and glomerular injury.

Keywords: anti-GBM glomerulonephritis; CD8; macrophage; monocyte; receptor; scavenger

Introduction

In previous work, severe crescentic glomerulonephritis (GN) was induced in WKY rats by administrating a small dose of anti-glomerular basement membrane (GBM) antiserum (25 μl containing 325 μg of rabbit IgG/100 g body weight). This model is characterized by massive accumulation of monocytes/macrophages (Mo/Mφ, ED-1+ cells) in the glomeruli and by a high frequency of crescent formation in glomeruli [1–3]. The unique features of this model may be due to the high susceptibility of WKY rats to anti-GBM antibody compared with other rat strains [1]. Anti-GBM rats produce a rapidly progressing GN, in which all animals become moribund within several weeks. Although the number of CD8+ cells in glomeruli was much less than that of Mo/Mφ, a crucial role for CD8+ cells in GN induction was demonstrated in CD8-depleted rats. In these animals, glomerular Mo/Mφ accumulation, crescent formation and severe proteinuria were almost entirely abolished [1]. Although these
findings indicate a crucial role for CD8⁺ cells in this model, a possible involvement of Mo/Mϕ cannot be excluded. Recently, we found that induction of crescentic GN was suppressed when monocyte chemoattractant protein 1 (MCP-1) was neutralized by multiple injections of monoclonal antibody against MCP-1, suggesting a role for Mo/Mϕ in glomerular injury and crescent formation [2].

In the present study, we examined correlations between Mo/Mϕ as well as CD8⁺ cell numbers and degree of glomerular injury to determine whether Mo/Mϕ or CD8⁺ cells play a direct role in glomerular injury and lesions. Further, to test involvement of Mo/Mϕ in anti-GBM GN more directly, we examined the effect of Mϕ depletion on the development of the anti-GBM GN. Liposome-encapsulated dichloromethylene diphosphonate (liposome-MDP) was introduced specifically to deplete Mϕ without affecting other leukocyte types, such as neutrophils, lymphocytes or Mo [4–8]. We treated WKY rats with liposome-MDP to deplete Mϕ after induction of anti-GBM GN. We found that both the amount of protein excreted in the urine and the percentage of crescentic glomeruli were strongly correlated with glomerular ED-1⁺ Mo/Mϕ numbers, but not with CD8⁺ cells. In addition, liposome-MDP treatment markedly suppressed lesion formation in this model, indicating a pivotal role for Mϕ in glomerular injury and crescent formation.

Subjects and methods

Induction of anti-GBM GN using different doses of anti-GBM antibody

Female inbred WKY rats, weighing 150–200 g, were obtained from Charles River Inc. (Atsugi, Kanagawa, Japan). Anti-GBM antiserum was prepared by immunizing rabbits with rat GBM solubilized with trypsin. In an initial experiment, rats were given the anti-GBM antibody at several doses: 5.0, 7.5, 10.0 and 25.0 μl/100 g body weight (three rats each). All animals were sacrificed at day 8 to examine correlations between the numbers of ED-1⁺ Mo/Mϕ or CD8⁺ cells per glomerular cross-section and the amount of urinary protein or the frequency of crescentic glomeruli.

Preparation of MDP-entrapped liposome (liposome-MDP)

MDP was kindly provided by Boehringer Mannheim GmbH (Mannheim, Germany) and was encapsulated in liposome as described previously [4]. In brief, phosphatidylcholine (Nippon Fine Chemicals, Osaka, Japan), diacetyl phosphate (Nakarai Tesque, Ltd, Kyoto, Japan) and cholesterol (Wako Chemical Co., Tokyo, Japan) were dissolved in chloroform (Nakarai Tesque, Ltd) in a round-bottom flask. A thin film was formed on the interior of the flask under reduced pressure while using a rotary. Thereafter, MDP dissolved in distilled water was dispersed on the film. After gentle shaking, MDP was entrapped in liposomes and the product was washed with phosphate-buffered saline (PBS) by centrifugation, then suspended in PBS, and extruded through a polycarbonate membrane (pore size of 0.8 μm, Millipore, Tokyo, Japan) to remove large liposome particles. Finally, the liposome-MDP was suspended in PBS at an approximate concentration of 150 mM MDP. Control liposome without MDP entrapment was also prepared.

Depletion of Mϕ

Rats were divided into three groups (six rats each). Two of the groups received intravenous (i.v.) 25 μl/100 g body weight of rabbit anti-GBM antibody [1], and one of these was then given liposome-MDP while the other was given control liposome (150 μmol/rat, i.v.) daily from day 3 to day 7 after the anti-GBM antibody injection. The third group was given 25 μl/100 g of normal rabbit serum followed by daily treatment with liposome-MDP from day 3 to day 7. Urine specimens were collected on days 3 and 8 by housing the animals in metabolic cages for 24 h. The amounts of protein excreted in the urine were determined using a protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). All animals were sacrificed at day 8 to obtain the kidneys. Peripheral blood was collected on days 3 and 8 from these rats, and the number of leukocytes in the circulation was counted.

Histology and immunohistochemistry

A portion of each kidney obtained on day 8 was fixed in methyl-Carnoy’s solution and embedded in paraffin. The paraffin-embedded tissues were sectioned at 4 μm thicknesses and stained with haematoxylin and periodic acid–Schiff reagent. The numbers of glomeruli with crescent formation were counted by observing >50 glomeruli in each section and calculating the frequency (%) of crescents. We defined a ‘cellular crescent’ as ‘a lesion consisting of proliferating epithelial and inflammatory cells filling part or all of Bowman’s space and that consist of at least two layers of cellular proliferation’ (definition 1) [9]. We also defined it as a ‘proliferative lesion in the Bowman’s spaces of >25% of the circumference’ (definition 2).

For immunohistochemistry, the paraffin-embedded sections were dewaxed and incubated sequentially with normal goat serum (1:20 dilution) for 20 min, with monoclonal antibodies against rat Mo/Mϕ (ED-1; Dainippon Seiyaku Co., Tokyo, Japan, 1:500 dilution) or CD8⁺ cells (MRC-OX8; PHS Centre for Applied Microbiology and Research, Wilsire, UK, 1:200 dilution) for 1 h, and with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (EnVision, Dako Japan Co., Kyoto, Japan) for 1 h. The peroxidase reaction product was visualized with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride–0.01% hydrogen peroxide as a substrate. The nuclei of cells stained with these antibodies were counted in >50 glomerular cross-sections in each kidney under a light microscope.

We previously reported that the amount of CD8⁺ cells that accumulated in the glomeruli of this model reached a peak at day 3 (10.1 ± 5.2/glomerular cross-section), whereas the amount of ED-1⁺ Mo/Mϕ increased steadily from 19.5 ± 7.6 at day 3 to 36.8 ± 11.4/glomerular cross-section at day 6 [1]. To examine whether ED-1⁺ cells infiltrating the glomeruli were Mo or Mϕ at days 1 and 3, the expression of class A scavenger receptor was examined as a Mϕ marker by using a monoclonal antibody (MSR-A: CD204, SRA-E5,
Macrophage in anti-GBM glomerulonephritis

Funakoshi Co., Ltd, Tokyo, Japan) [10]. The paraffin-embedded sections were dewaxed and heated for 10 min by microwave in 0.01 M sodium citrate buffer (pH 2.0) to retrieve antigens. Then, they were incubated with the antibody (10 μg/ml) to identify the scavenger receptor by immunohistochemistry as described above. To determine whether the glomerular CD8+ cells bear ED-1, serial sections of the kidneys obtained at day 8 were immunostained.

Blood samples were collected on days 3 and 8 to determine total leukocyte counts. To examine percentages of Mo/Mϕ and CD8+ cells in blood, smear samples were immunostained with anti-rat Mo/Mϕ (ED-1) and CD8 antibodies. The data were then presented as a percentage of ED-1- or OX-8-positive cell counts/total nucleated cell counts on each blood smear.

Serum samples were collected on days 3 and 8 to examine serum creatinine and blood urea nitrogen (BUN) concentrations (mg/dl) using a modified Jaffe and urease-indophenol methods (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

Glomerular expression of ICAM-1 and MCP-1 mRNA

Glomeruli were isolated individually from renal cortex by a standard sieving method that yielded a 89–94% purity, and total cellular RNA was extracted from the glomeruli by a modified acid-phenol–guanidium thiocyanate method (TRIzol, Gibco-BRL, Grand Island, NY). The RNase protection assay was employed to detect glomerular dehydrogenase (GAPDH) mRNA expression of intercellular adhesion molecule 1 (ICAM-1), MCP-1 and glyceraldehyde-3-phosphate as described previously [2,3]. For quantitation of the expression of each mRNA, the autoradiography films were scanned in an optical scanner (JX-330M, Sharp, Osaka, Japan) and the density of each protected band was quantified by image analysis software (NIH image, National Institutes of Health, Bethesda, MD). The data were represented as the ratio of specific mRNA/GAPDH mRNA band density to ensure a constant quantity of mRNA in each sample.

Statistical analysis

The data were expressed as means±SD. Statistical significance was analysed by the Mann–Whitney U-test. Differences with P<0.05 were considered statistically significant. Correlations between the amount of urinary protein (mg/day) or percentage of crescentic glomeruli (%) and the number of glomerular ED-1+ cells or CD8+ cells were analysed by the Pearson’s correlation coefficient method.

Results

Correlations between glomerular leukocyte counts and injury

Administration of 25 μl of anti-GBM antibody per 100 g body weight induced marked proliferative GN that included elevation in serum creatinine from 0.54±0.1 to 2.8±0.1 mg/dl and in BUN from 20.0±1.9 to 63.2±8.9 mg/dl at day 8. Increasing doses of anti-GBM antibody caused increases in the number of ED-1+ Mo/Mϕ and CD8+ cells that accumulated in the glomeruli. To determine whether Mo/Mϕ or CD8+ cells were more intensively involved in induction of glomerular injury, we analysed correlations between the numbers of glomerular Mo/Mϕ or CD8+ cells and the amounts of urinary protein or frequency of glomerular crescent formation in these animals.

As shown in Figure 1, the amount of urinary protein correlated significantly with both the numbers of glomerular ED-1+ Mo/Mϕ (r=0.965, P<0.0001) and CD8+ cells (r=0.639, P<0.05). The percentage of crescentic glomeruli also correlated with the numbers of both glomerular ED-1+ cells (r=0.894, P<0.0001) and CD8+ cells (r=0.628, P<0.05). However, the number of glomerular ED-1+ Mo/Mϕ was more strongly correlated with proteinuria and crescent formation, suggesting that Mo/Mϕ may be involved in induction of glomerular injury and lesion.

Histology and immunohistochemistry

Marked accumulation of mononuclear leukocytes in glomeruli was found 8 days after anti-GBM antibody injection (Figure 2). Most of these glomeruli had accompanying cellular crescents, severe necrotizing lesions and exudation of proteinaceous materials in the Bowman’s space. These intra- and extracapillary

![Fig. 1. Correlations between the amount of urinary protein (mg/day) (A) or the frequency of crescentic glomeruli (B) with the numbers of glomerular ED-1+ Mo/Mϕ or CD8+ cells. There was a stronger correlation between urinary protein excretion and the numbers of glomerular Mo/Mϕ (r=0.965, P<0.0001) than with numbers of CD8+ cells (r=0.639, P=0.0323) (A). There was also a stronger correlation between the percentage of crescentic glomeruli and the number of glomerular Mo/Mϕ (r=0.894, P<0.0001) than with numbers of CD8+ cells (r=0.628, P=0.0367) (B).](https://academic.oup.com/ndt/article-abstract/19/12/2997/1807764/1807764?rss=true)
glomerular lesions were almost completely suppressed by the liposome-MDP treatment. There were no glomerular lesions in control animals injected with liposome-MDP and normal rabbit serum.

The frequency of crescentic glomeruli defined as a lesion consisting of proliferating epithelial and inflammatory cells, which fills part or all of Bowman’s space and that consists of at least two layers of cellular proliferation (definition 1), was significantly decreased from 91.3±2.7 to 23.3±7.6% (∕₀.₀₁) by the liposome-MDP treatment. The frequency of glomeruli accompanied by extracapillary proliferative lesions along >25% of Bowman’s capsule (definition 2) was also significantly decreased by the treatment, from 70.6±2.1 to 10.2±3.3% (∕₀.₀₁).

The number of ED-1⁺ Mo/Mφ infiltrating to glomeruli was 32.1±1.2 cells/glomerular cross-section in the anti-GBM antibody-injected rats at day 8 (Figure 3), which was also significantly reduced to 1.4±0.3 cells/glomerular cross-section by the liposome-MDP treatment (∕₀.₀₁). This number was still significantly larger than that of rats given normal rabbit serum and the liposome-MDP (0.6±0.1 cells/glomerular cross-section, ∕₀.₀₁).

The number of CD8⁺ cells accumulating in the glomeruli was 5.4±0.7 cells/glomerular cross-section in rats injected with the anti-GBM antibody and control liposome at day 8 (Figure 3). The glomerular accumulation of CD8⁺ cells was significantly reduced to 0.5±0.1 cells/glomerular cross-section by the liposome-MDP treatment (∕₀.₀₁, Figure 4e). This number was comparable with that of rats given normal rabbit serum and liposome-MDP (0.3±0.1 cells/glomerular cross-section).

To determine whether ED-1⁺ cells accumulated in glomeruli on days 1 and 3 were Mφ or Mo, immunodetection of scavenger receptor class A was examined. As seen in Figure 4, most of the ED-1⁺ cells

---

**Fig. 2.** Mesangial proliferation, severe necrotizing lesions and marked crescent formation were observed at day 8 after injection of anti-GBM antibody (A and C). In liposome-MDP-treated rats, proliferative glomerular lesions and crescent formation were almost completely suppressed at day 8 (B and D). Magnification: (A) and (B) ×120; (C) and (D) ×500.
accumulating in the glomeruli at day 3 bore the Mϕ marker, whereas the ratio was low in the glomeruli at day 1. This finding indicated that the ED-1⁺ cells were Mϕ when the liposome-MDP administration was started.

The liposome-MDP treatment did not affect the total number of leukocytes or the differential fractions of Mo and CD8⁺ cells in the circulation (total peripheral leukocyte counts were 8620 ± 340/mm³ and 8200 ± 320/mm³ in rats before and after administration of normal rabbit serum and the liposome-MDP, respectively). The leukocyte count was not changed at day 8 in rats given the anti-GBM antibody and liposome-MDP (8160 ± 360/mm³). The percentages of ED-1⁺ Mo and CD8⁺ cells in the peripheral blood were 16.7 ± 5.5 and 20.5 ± 4.0%, respectively, before the experiment and were unchanged after the liposome-MDP injection (Mo, 14.8 ± 4.2%, \( P > 0.05 \); CD8⁺ cells, 23.2 ± 6.2%, \( P > 0.05 \)).

Immunohistochemistry using serial sections was used to examine co-expression of ED-1⁺ and CD8⁺ cells. The immunodetected ED-1⁺ cells were localized at sites different from where CD8⁺ cells were present in the sequential serial sections. No distinct ED-1⁺ cells were demonstrated to be positive for CD8 (Figure 5).

Detection of glomerular ICAM-1 and MCP-1 mRNA

To evaluate the glomerular inflammatory response semi-quantitatively, the expression of ICAM-1 and MCP-1 mRNA was examined in glomeruli at day 8. The amounts of both ICAM-1 and MCP-1 mRNA were increased by antibody injection, and liposome-MDP treatment suppressed the increase in glomerular ICAM-1 by 53.3% and reduced the increase in MCP-1 mRNA expression by 86.7% (Figure 6). The expression of ICAM-1 and MCP-1 mRNA was negligible in the glomeruli of rats given normal rabbit serum and the liposome-MDP.

Urinary protein excretion

After injection of the anti-GBM antibody, all rats excreted substantial amounts of protein in the urine at day 8 (103.8 ± 19.8 mg/day). The proteinuria was significantly reduced by the liposome-MDP treatment (31.8 ± 15.9 mg/day, \( P < 0.01 \)), although it remained greater than in rats given normal rabbit serum and the liposome-MDP (11.7 ± 3.2 mg/day, \( P < 0.01 \)).
Discussion

In the present study, we demonstrated that anti-GBM-induced accumulation of Mφ in glomeruli was reduced successfully by liposome-MDP administration in WKY rats. This reduction was accompanied by a marked suppression of crescent formation and urinary protein excretion. Mφ have been observed in various inflammatory tissues, and the number of Mφ at inflammatory sites has been shown to correlate with severity of tissue injuries [11]. Crescentic GN is a type of glomerular disease which is generally considered to progress rapidly to end-stage kidney failure in humans. Since glomerular accumulation of Mφ has been demonstrated in human crescentic GN, these cells are thought to play a pivotal role in the development of crescentic formation [12,13]. Although these findings indicate that Mφ are involved in glomerular injuries, a role for Mφ in tissue repair has also been proposed. Mφ may remove tissue debris and stimulate proliferation and renewal of tissue cells at inflammatory sites [14]. Thus, the various roles that Mφ play in inflammatory processes remains controversial and may differ according to inflammation types or to the time points during the course of diseases. Although attempts have been made to examine Mφ involvement in several GN models [2,15,16], clear conclusions have not yet been reached. Part of the problem has been a lack of specific and efficient methods to interfere with participation of Mφ in diseases in vivo. Recent studies showing evidence for participation of Mφ in accelerated anti-GBM GN demonstrated that adoptive transfer of Mφ caused an increase in urinary protein and stimulated mesangial cell proliferation [17]. The present study demonstrated that glomerular histology and injury were significantly attenuated by Mφ depletion in a different animal model, which also indicated an important role for Mφ. The pathophysiology of the anti-GBM GN model in WKY rats in the present study is unique and different from that of other conventional anti-GBM nephritis models [1]. Since crescentic GN progresses to lethal renal failure within a few weeks, accumulation of Mφ in glomeruli during later phases of this model has also been thought to cause disease deterioration.

In a previous study, we found that suppression of glomerular Mo/Mφ infiltration by neutralization of MCP-1 using a specific anti-MCP-1 antibody reduced...
urinary protein excretion and reduced the development of crescent formation, indicating that Mϕ may cause direct glomerular injury [2]. Since Mϕ have been identified as a component of the cell population in crescentic lesions, these cells have been thought to induce crescent formation [12,13]. The present study provides the first evidence for an important role for Mϕ in glomerular injury and crescent formation during anti-GBM GN in WKY rats.

Administration of liposome-MDP was introduced as an efficient and selective method to kill Mϕ in vitro and in vivo without affecting other leukocyte populations [4–8]. Treatment efficiency was confirmed in the present study by suppression of the accumulation of Mϕ in glomeruli. Liposome-MDP has been shown to exert its cytotoxic effect on Mϕ after engulfment. We previously demonstrated that phagocytosed liposome-MDP caused apoptosis of Mϕ [6]. Following treatment with liposome-MDP, the number of ED-1+ cells accumulating in glomeruli decreased whereas the number of these cells in the circulation was unaffected, indicating that most of the glomerular ED-1+ cells were Mϕ and that circulating ED-1+ cells were Mo. This was confirmed by using immunostaining of anti-Mϕ scavenger receptor class A as a Mϕ marker, which showed that only a small population of ED-1+ cells was positive for the marker at day 1, whereas most ED-1+ cells bore the marker at day 3. Circulating Mo appear to be attracted to the glomeruli where they are differentiated to Mϕ by various stimuli such as cytokines [18]. These Mϕ are able to engulf liposome-MDP in the glomeruli and then cause apoptosis. The present study showed that depletion of Mϕ in the glomeruli suppressed crescent formation and reduced the amount of urinary protein, which strongly suggests that Mϕ cause direct injury to glomerular structure and function. A crucial role for Mϕ in tissue injury has also been demonstrated in other disease models such as in experimental allergic encephalomyelitis [4] and arthritis [5]. In these models, liposome-MDP-induced depletion of Mϕ caused inhibition of tissue injury.

Interestingly, the number of CD8+ cells in the glomeruli was also reduced to near zero levels by liposome-MDP treatment. This contrasts with previous findings, which showed that liposome-MDP administration had no effect on recruitment of CD8+ cells [7]. However, CD8+ cell involvement may have been different since the previous experiment used a completely different disease model. Nevertheless, if CD8+ cells were...
cells represent a subset of Mϕ, as was reported recently [19], a reduction in CD8+ cells caused by liposome-MDP is possible. However, the immunostaining using serial sections in the present study showed that none of the CD8+ cells accumulating in the glomeruli at day 8 definitely bore ED-1. Since liposome-MDP treatment did not reduce CD8+ cells in the circulation, it is possible that the reductions in glomerular CD8+ cells were not caused by direct cytotoxicity of liposome-MDP on CD8+ cells but indirectly by a reduction in glomerular Mϕ accumulation. Although the glomerular Mϕ are thought to play a role in the accumulation of CD8+ cells in glomeruli, we demonstrated that CD8+ cells regulated accumulation of Mϕ in the glomeruli [1]. Taken together, these findings indicate that both Mϕ and CD8+ cells stimulate or cooperate with each other and play an important role in the induction and progression of anti-GBM nephritis in WKY rats.

Clinical trials using liposome-MDP have been already been initiated in patients having several disease types [20]. Inhibition of Mϕ activity may be a useful therapeutic strategy in human GN characterized by glomerular Mϕ accumulation.

In summary, we demonstrated that the liposome-MDP treatment caused almost complete suppression of Mϕ accumulating in the glomeruli from WKY rats with anti-GBM nephritis, and that this treatment concomitantly reduced urinary protein excretion and frequency of glomerular crescent formation. These results suggest that glomerular Mϕ play a pivotal role in the development of glomerular injury in this model, presumably through direct effects of Mϕ causing glomerular structure injury and through indirect effects that stimulate recruitment of CD8+ cells in the glomerulus.

Acknowledgements. We thank Kan Yoshida for his excellent technical assistance with sectioning the specimens. A part of this work was supported by a Grant-in-aid from the Japanese Ministry of Education, Culture and Sport (13470209).

Conflict of interest statement. None declared.

References

Accepted in revised form: 6.7.04

Received for publication: 30.1.04

Downloaded from https://academic.oup.com/ndt/article-abstract/19/12/2997/1807764 by guest on 11 November 2018