Differential Roles of Beta2 Integrins in Human Crescentic Glomerulonephritis

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Differential Roles of Beta2 Integrins in Human Crescentic Glomerulonephritis

Tetsuya OOTAKA[1], Hiroshi SATO[2], Toshinobu SATO[3], Sadayoshi ITO[2] and Takao SAITO[4]

[1]*Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Tohoku University, Sendai, Japan*
[2]*The Second Department of Internal Medicine, Tohoku University School of Medicine, Sendai, Japan*
[3]*Department of Blood Purification, Tohoku University Hospital, Sendai, Japan*
[4]*The Fourth Department of Internal Medicine, Fukuoka University School of Medicine, Fukuoka, Japan*

半月体性腎炎における beta2 integrin 関与の病型間の相違について

大高徹也[1], 佐藤　博[2], 佐藤寿伸[3], 伊藤貞嘉[2], 斉藤喬雄[4]

[1]東北大学医学部保健学科　検査技術科学専攻
[2]東北大学医学部　第2内科
[3]東北大学病院　血液浄化療法部
[4]福岡大学医学部　第4内科

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Running Title: Leukocyte integrins in crescentic glomerulonephritis

An immunohistochemical study was performed to investigate the involvement of beta2 integrins in the glomerular infiltration of leukocytes in different types of human crescentic glomerulonephritis (CrGN). On 51 ethanol-fixed paraffin embedded renal biopsy specimens (34 cases of pauci-immune type and 17 cases of immune-mediated type), the following antigens were evaluated by immunoperoxidase method ; the glomerular (intra-capillary and extra-capillary) expression of ICAM-1, deposition of FRA and infiltration of leukocytes bearing beta2 integrins (LFA-1, CR3 and CR4).

ICAM-1 was expressed both in extra-capillary and intra-capillary areas in very early stage of crescent formation and was rapidly decreased with the progression of the stage of crescent. Similarly, infiltration of leukocytes bearing beta2 integrins were observed both in extra-capillary and intra-capillary areas in early stage of crescent formation and were rapidly decreased with the progression of the stage of crescent. In pauci-immune type, the extra-capillary expression of ICAM-1 was significantly correlated with extra-capillary infiltration of LFA-1+ cells ($r=0.800$, $p<0.0001$) and CR3+ cells ($r=0.791$, $p<0.0001$). The extra-capillary deposition of FRA was also significantly correlated with extra-capillary infiltration of CR3+ cells ($r=0.685$, $p<0.001$) and CR4+ cells ($r=0.741$, $p<0.0001$). In both types, the intra-capillary expression of ICAM-1 was significantly correlated with intra-capillary infiltration of LFA-1+ cells ($r=0.618$, $p<0.01$ and $r=0.754$, $p<0.05$ respectively).

These results suggested that ICAM-1/LFA-1 and ICAM-1/CR3 interaction might be involved in

Correspondence to Tetsuya Ootaka MD, Department of Medical Technology, School of Health Sciences, Faculty of Medicine, Tohoku University, 2-1, Seiryo-cho, Aoba-ku, Sendai, 980-8575, Japan.
e-mail : totaka@mail.tains.tohoku.ac.jp Phone : +81-22-717-7952

both the extra- and intra-capillary infiltration of leukocytes in pauci-immune CrGN. CR3 and CR4 might also function as fibrin/fibrinogen receptors in extra-capillary lesions in this type. On the other hand, only ICAM-1/LFA-1 interaction might be involved only in the intra-capillary infiltration of leukocytes in immune mediated CrGN.

Introduction

Crescentic glomerulonephritis (CrGN) is one of the most important glomerular diseases as a cause of end-stage renal disease because of its rapidly progressive course[1)-4)]. Cell-mediated mechanism is considered to be important in the induction of glomerular injury in various kinds of renal diseases including CrGN[5)-9)]. Among several mechanisms known to be involved in the glomerular infiltration of leukocytes, many studies have shown the importance of the interaction of intercellular adhesion molecule-1 (ICAM-1) and leukocyte function associated antigen-1 (LFA-1)[10)-16)]. We have reported the differential roles of beta2 integrins in several kinds of glomerular diseases including membranoproliferative glomerulonephritis type I (MPGN), IgA nephropathy (IgAN) and Henoch-Schoenlein purpura nephritis (HSPN)[15)-17)]. However, it is still unclear whether beta2 integrins might play differential roles in different subtypes of human CrGN, i.e., pauci-immune type and immune-mediated type (the latter subdivided into anti-GBM type and immune-complex type).

To investigate the involvement of beta2 integrins in the glomerular infiltration of leukocytes in individual subtypes of human CrGN, we evaluated the expression of intercellular adhesion molecules-1 (ICAM-1), the deposition of fibrin/fibrinogen related antigens (FRA) in

Table 1. Clinical findings of patients at renal biopsy

	Pauci-immune CrGN (34)	Immune-mediated CrGN (17)
Final Diagnosis	Idiopathic CrGN (26) Periarteritis Nodosa (7) Wegener's Granulomatosis (1)	anti-GBM CrGN (3) immune complex CrGN (8) IgA nephropathy (5) Lupus Nephritis (1)
Age (years)	62.3±1.7*	57.3±3.5
Sex (M : F)	15 : 19	7 : 10
Urine Protein (g/day)	1.9±0.4	3.5±0.7
Urine RBC		
0-4 (−)	2 cases	0 case
5-19 (+)	3 cases	0 case
20-49 (++)	5 cases	2 cases
50-99 (+++)	17 cases	12 cases
100- (++++)	7 cases	3 cases
BUN (mg/dl)	51.9±4.7	49.8±6.2
Cr (mg/dl)	4.3±0.5	4.1±0.7

*: mean±SEM

Table 2. Antibodies used for immunocytochemical study

Antigen	CD number	Supplier	Clone
Beta2 integrins			
LFA-1	CD11a	Dakopatts	MHM24
Mac-1 (CR3)	CD11b	Dakopatts	2LPM19c
p150, 95 (CR4)	CD11c	Dakopatts	KB90
Adhesion Molecule			
ICAM-1	CD54	Dakopatts	6.5B5

glomeruli and infiltration of leukocytes bearing beta2 integrins.

Methods

Materials :

From January 1991 to may 1997, 51 renal biopsy specimens were obtained from patients with histological diagnosis of crescentic glomerulonephritis or focal necrotizing glomerulonephritis and with clinical diagnosis of rapidly progressive glomerulonephritis (41 cases), periarteritis nodosa (7 cases), Wegener's granulomatosis (1 case) or systemic lupus erythematosus (1 case). These cases were divided into the following two groups according to the routine immunohistological findings; pauci-immune crescentic glomerulonephritis (pauci-immune CrGN ; 34 cases) and other (immune-mediated) type of crescentic glomerulonephritis (immune-mediated CrGN ; 17 cases). The final diagnosis and clinical findings of these two groups are shown in Table 1. These two groups were not significantly different with each other in these clinical data. For light microscopy and immunohistological study, all biopsy specimens were fixed in 95% cold ethanol (4°C, 24 hr.), dehydrated and embedded in paraffin at 56°C[18].

Antibodies and immunohistochemical method :

The following antisera were obtained from Dakopatts (Glostrup, Denmark) : rabbit antisera to human IgG, IgA, IgM, C1q, C3c and fibrin/fibrinogen-related antigens (FRA). The mouse monoclonal antibodies used for immunocytochemical staining were shown in Table 2. The immunohistochemical staining for immune deposits was performed by two-layer indirect immunoperoxidase method and immunocytochemical staining was performed by three-layer indirect immunoperoxidase method as described before[18][19]. The second and third antibodies were obtained from Dakopatts (Glostrup, Denmark) as follows : horseradish peroxidase (HRP)-conjugated or non-conjugated rabbit anti-mouse immunoglobulins, HRP-conjugated swine anti-rabbit immunoglobulins and alkaline phosphatase (AP)-conjugated goat anti-mouse/rabbit immunoglobulins.

The double immunostaining study was performed to examine the co-localization of the infiltration of leukocytes bearing beta2 integrins with the expression of ICAM-1 or the deposition of FRA. In the first step, the peroxidase activity was demonstrated brown by DAB after immuno-staining for the infiltrating leukocytes by the use of HRP-labelled final antibody. In the second step, the alkalline phos-

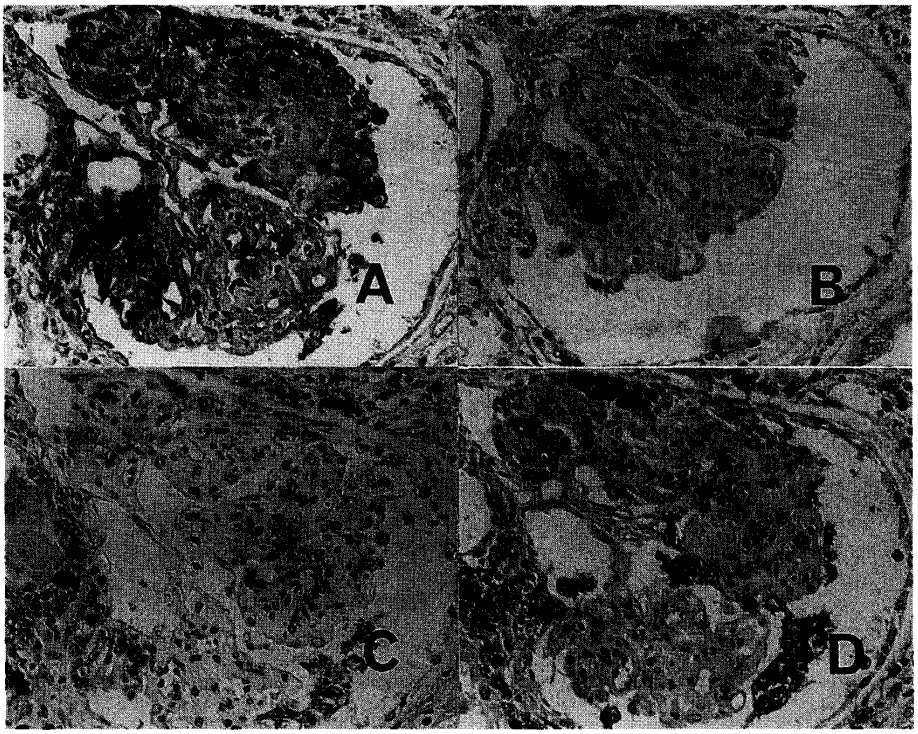

Figure 1. Micrographs of a glomerulus in early stage of pauci-immune crescentic glomerulonephritis by immunoperoxidase staining showing the expression of ICAM-1 (A) and the infiltration of LFA-1+ (B), CR3+ (C) and CR4+ immune cells (D) (counterstained with hematoxylin, magnifications ×400).

phatase activity was demonstrated by new fuchsin after immuno-staining for ICAM-1 or FRA by the use of AP-labelled final antibody.

Immunohistological analysis

The number of positively stained extra- and intra-capillary cells was expressed as mean cell number/glomerular cross section (GCS). The staining intensities of glomerular deposition of FRA and of expression of ICAM-1 in the extra- and intra-capillary areas were evaluated semi-quantitatively from (−) to (3+) and expressed as mean value of all glomeruli in each case. The semi-quantitative evaluation was performed by two independent pathologists (JS and TO).

Light microscopy

The ethanol-fixed specimens were also stained with hematoxylin and eosin (HE) and periodic acid Schiff (PAS). The extent of glomerular injury in each case was evaluated by the index of glomerular lesion (IGL), a semi-quantitative scoring system introduced by Suwa and Takahashi[8]. The percentage of the number of cellular crescents (%CC), fibrocellular crescents (%FCC) and fibrous crescents (%FC) were also recorded. The activity index of crescent formation (AICF) was defined and calculated as follows:

$$AICF = 3 \times (\%CC) + 2 \times (\%FCC) + 1 \times (\%FC)$$

Clinical data

The following laboratory examinations were also performed at the time of renal biopsy: 24-hour urinary protein (UP), urinary red blood cells (URBC), blood urea nitrogen (BUN),

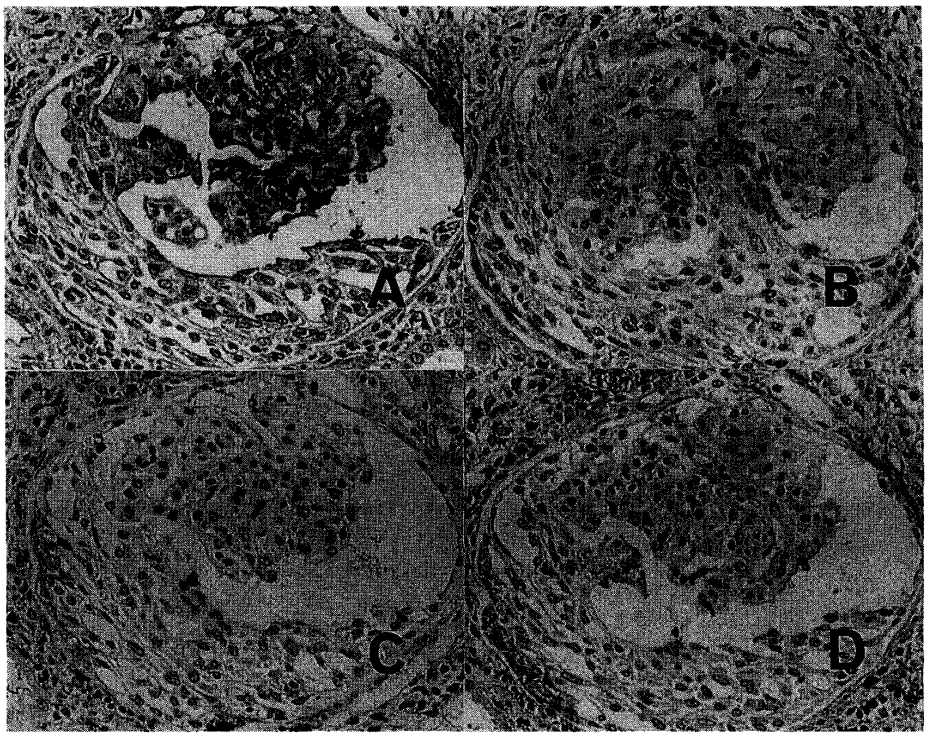

Figure 2. Micrographs of a glomerulus in late stage of pauci-immune crescentic glomerulonephritis by immunoperoxidase staining showing the expression of ICAM-1 (A) and the infiltration of LFA-1+ (B), CR3+ (C) and CR4+ immune cells (D). These integrin+ immune cells are obviously decreased together with the decrease of ICAM-1 expression. (counterstained with hematoxylin, magnifications ×400)

serum creatinine (Cr) and C-reactive protein (CRP). URBC was scored semi-quantitatively from (−) to (4+).

Statistical analysis

The correlations between clinical, histological and immunohistological data was evaluated by Spearman's rank correlation coefficients according to non-parametric nature of these data. A difference in these data between two groups was evaluated by Mann-Whitney's U-test or Wilcoxon's rank sum test. P values were adjusted by the method of Bonferroni to correct for the inflation of type I error introduced by multiple analyses. Corrected P values less than 0.05 were considered significant.

Results

Early stage of crescent formation in pauci-immune CrGN (Fig. 1A, B, C, D):

In early stage of extra-capillary proliferation with celullar crescent, ICAM-1 was strongly expressed (Fig. 1A) and FRA was strongly deposited on proliferating extra-capillary cells in Bowman's space. Large number of leukocytes bearing LFA-1 (Fig. 1B), CR3 (Fig. 1C) and CR4 (Fig. 1D) were also found in this area.

ICAM-1 was also strongly expressed in intra-capillary area in this stage of crescent formation. Leukocytes bearing LFA-1, CR3 and CR4 were also found in this area.

Table 3. Spearman's rank correlation coefficients of extra-capillary expression of ICAM-1 or deposition of FRA with extra-capillary infiltration of leukocytes bearing beta2 integrins

pauci-immune CrGN	LFA-1 (CD11a)	CR3 (CD11b)	CR4 (CD11c)
ICAM-1	0.800**	0.791**	0.831**
FRA	0.675*	0.685*	0.741**
immune-mediated CrGN	LFA-1 (CD11a)	CR3 (CD11b)	CR4 (CD11c)
ICAM-1	0.438	0.212	0.474
FRA	-0.070	0.250	0.090

*: $p<0.001$, **: $p<0.0001$ after Bonferroni's correction

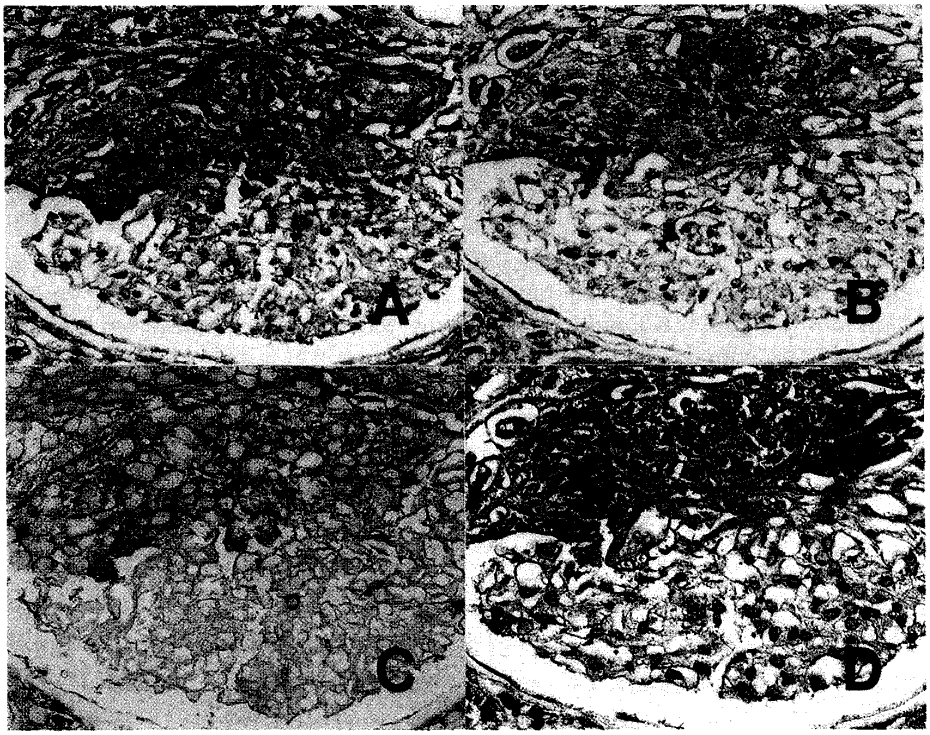

Figure 3. The double immuno-staining for the expression of ICAM-1 or the deposition of FRA (stained in pink color) and the infiltration of LFA-1+, CR3+ or CR4+ leukocytes (stained in brown color). The expression of ICAM-1 (pink) was co-localized with the infiltration of LFA-1+ leukocytes (brown) (A) and with CR3+ leukocytes (brown) (B). The deposition of FRA (pink) was also co-localized with the infiltration of CR3+ leukocytes (brown) and CR4+ leukocytes (brown). (counterstained with hematoxylin, magnifications ×400)

Late stage of crescent formation in pauci-immune CrGN (Fig. 2A, B, C, D):

In late stage of extra-capillary prolifreration with fibrocellular or fibrous crescent, ICAM-1 expression (Fig. 2A) and FRA deposition on the crescent was obviously diminished. The numbers of leukocytes bearing LFA-1 (Fig. 2B), CR3 (Fig. 2C) and CR4 (Fig. 2D) were also decreased in this area.

ICAM-1 expression was diminished in

Table 4. Spearman's rank correlation coefficients of intra-capillary expression of ICAM-1 or deposition of FRA with intra-capillary infiltration of leukocytes bearing beta2 integrins

pauci-immune CrGN	LFA-1 (CD11a)	CR3 (CD11b)	CR4 (CD11c)
ICAM-1	0.618**	0.499*	0.456*
FRA	−0.104	−0.169	0.086
immune-mediated CrGN	LFA-1 (CD11a)	CR3 (CD11b)	CR4 (CD11c)
ICAM-1	0.754*	0.612	0.746*
FRA	0.278	0.284	0.368

*: $p<0.05$, **: $p<0.01$ after Bonferroni's correction

Table 5. Spearman's rank correlation coefficients of extra-capillary proliferative changes with extra-capillary infiltration of leukocytes bearing beta2 integrins

pauci-immune CrGN	cellular crescent (%)	fibrocellular crescent (%)	fibrous crescent (%)	activity index of crescent
LFA-1	0.604**	0.391	-0.610**	0.640**
CR3	0.528*	0.494	−0.548*	0.659**
CR4	0.601**	0.543*	−0.569*	0.743***
immune-mediated CrGN	cellular crescent (%)	fibrocellular crescent (%)	fibrous crescent (%)	activity index of crescent
LFA-1	0.300	0.401	−0.332	0.443
CR3	0.231	0.328	−0.041	0.304
CR4	0.252	0.001	-0.017	0.304

*: $p<0.05$, **: $p<0.01$, $p<0.001$ after Bonferroni's correction

intra-capillary areas in this stage of crescent formation. Leukocytes bearing LFA-1, CR3 and CR4 were also found in this area in smaller number than the glomeruli in early stage.

Correlation of ICAM-1 expression and FRA deposition with infiltrating leukocytes bearing beta2 integrins in CrGN

Extra-capillary areas (Table 3, Fig. 3) :

Both the expression of ICAM-1 and the deposition of FRA in extra-capillary areas were significantly correlated with the extra-capillary infiltration of LFA-1+, CR3+ and CR4+ leukocytes in pauci-immune CrGN (Table 3). The double immunostaining revealed the co-localization of the expression of ICAM-1 with the infiltration of LFA-1+ leukocytes (Fig. 3A) and CR3+ leukocytes (Fig. 3B). Also, the deposition of FRA was co-localized with the infiltration of CR3+ leukocytes (Fig. 3C) and CR4+ leukocytes (Fig. 3D). On the other hand, either the expression of ICAM-1 or the deposition of FRA was not correlated with the extra-capillary infiltration of leukocytes bearing beta2 integrins in immune-mediated CrGN (Table 3).

Intra-capillary areas (Table 4) :

The expression of ICAM-1 in intra-capillary areas was significantly correlated with the intra-capillary infiltration of LFA-1+, CR3+

Table 6. Spearman's rank correlation coefficients of clinical findings with extra-capillary infiltration of leukocytes bearing beta2 integrins

pauci-immune CrGN	urinary protein (g/day)	urinary RBC	serum Cr (mg/dl)	CRP
LFA-1	−0.309	0.350	0.377	0.603**
CR3	−0.178	0.319	0.382	0.537*
CR4	−0.172	0.444	0.506*	0.554*
immune-mediated CrGN	**urinary protein (g/day)**	**urinary RBC**	**serum Cr (mg/dl)**	**CRP**
LFA-1	−0.117	0.171	−0.065	−0.172
CR3	−0.190	0.256	−0.006	−0.055
CR4	−0.291	0.419	−0.141	0.037

*: $p<0.05$, **: $p<0.02$ after Bonferroni's correction

and CR4+ leukocytes in pauci-immune CrGN. But the intra-capillary deposition of FRA was not correlated with the infiltration of these leukocytes bearing beta2 integrins in this type. The expression of ICAM-1 in intra-capillary areas was also significantly correlated with the intra-capillary infiltration of LFA-1+ and CR4+ leukocytes in immune-mediated CrGN. The intra-capillary deposition of FRA was not correlated with the infiltration of these leukocytes bearing beta2 integrins also in this type.

Correlation of the extra-capillary infiltration of leukocytes bearing beta2 integrins with the crescent formation in CrGN (Table 5)

All of the extra-capillary infiltration of LFA-1+, CR3+ and CR4+ leukocytes were significantly correlated with the percentage of the number of cellular crescents (%CC) and was inversely correlated with the percentage of the number of fibrous crescents (%FC) in pauci-immune CrGN. The activity index of crescent formation (AICF) was significantly correlated with the extra-capillary infiltration of leukocytes bearing beta2 integrins. On the other hand, none of the extra-capillary infiltration of these leukocytes was significantly correlated with any of %CC, %FCC, %FC or AICF in immune mediated CrGN.

Correlation of the extra-capillary infiltration of leukocytes bearing beta2 integrins with the clinical findings at renal biopsies (Table 6)

In pauci-immune CrGN, the value of CRP was significantly correlated with all of the extra-capillary infiltration of LFA-1+, CR3+ and CR4+ leukocytes. Serum Cr was also significantly correlated with the extra-capillary infiltration of CR4+ leukocytes. In immune mediated CrGN, on the other hand, none of the clinical data examined in this study was significantly correlated with any of the extra-capillary infiltration of these leukocytes.

Discussion

Crescentic glomerulonephritis (CrGN) is one of the most important glomerular diseases as a cause of end-stage renal disease because of its rapidly progressive course[1)-4)]. It is also known that the glomerular infiltration of immune cells is important in the early stage of human crescentic glomerulonaphritis. Patey et al. investigated in the expression of adhesion molecules, beta1 and beta3 integrins in

crescents in several kinds of human glomerulonephritis and demonstrated in crescents of early stage the expression of ICAM-1, VCAM-1, beta1 and beta3 integrins and LFA-1 and showed the importance of ICAM-1/LFA-1 interaction in the formation of crescents[14]. The importance of ICAM-1/LFA-1 interaction was also demonstrated in experimental crescentic glomerulonephritis[20)-23)]. Nishikawa et al showed the importance of ICAM-1/LFA-1 interaction in the glomerular infiltration of T-cells, monocytes and macrophages and in the formation of crescents in rat anti-GBM glomerulonephritis[21]. Coers reported that IFNγ released from T-cells induce ICAM-1 expression on glomerular epithelial cells in experimental pauci-immune CrGN[24]. Moutabarrik reported that IL1beta released from macrophages induce ICAM-1 expression on glomerular epithelial cells in vitro[25].

We have recently reported differential roles of beta2 integrins in individual glomerular diseases. LFA-1 was involved in the glomerular infiltration of leukocytes as a ligand of ICAM-1 in IgAN[15] and HSPN[16], while CR3 and CR4 were involved in the glomerular infiltration of leukocytes as complement receptors in MPGN[17]. However, the beta2 integrin-mediated mechanism of glomerular infiltration of leukocytes is still unclear in human CrGN, which is composed of several glomerular diseases of different pathogenic mechanisms, i.e., pauci-immune type, anti-GBM type and immune complex type. The beta2 integrins, which consist of three molecular types, are mainly expressed on leukocytes and have different spectrum of functions. According to their functions, they are called leukocyte function associated antigen-1 (LFA-1, CD11a/CD18), complement receptor 3 (CR3, CD11b/CD18) and CR4 (CD11c/CD18). ICAM-1, which are expressed mainly on endothelial cells, epithelial cells and leukocytes, is the major ligand for LFA-1, and also functions as a ligand for CR3. Both CR3 and CR4 have binding sites for C3b inactivator-cleaved C3b (C3bi) and fibrin/fibrinogen[26)-29)].

In pauci-immune CrGN, ICAM-1 was strongly expressed and numerous leukocytes bearing beta2 integrins were infiltrated in extra- and intra-capillary area in early stage of extra-capillary proliferation (cellular crescents). The expression of ICAM-1 and the infiltration of beta2 integrin+ leukocytes was diminished in late stage of extra-capillary proliferation (fibrocellular and fibrous crescents). These parameters were significantly correlated with the histological and clinical parameters which indicate the activity of this disease.

The intra- and extra-capillary expression of ICAM-1 was significantly correlated with the intra- and extra-capillary infiltration of LFA-1+ and CR3+ leukocytes respectively. These results were compatible with the previous observations reported by several investigators in crescent formation in several human glomerulonephritis and suggested that ICAM-1/LFA-1 and ICAM-1/CR3 interaction are involved in both the extra- and intra-capillary infiltration of leukocytes in active stage of human pauci-immune CrGN.

The extra-capillary deposition of FRA was significantly correlated with the extra-capillary infiltration of CR3+ and CR4+ leukocytes. It is considered that C3bi/CR3 or C3bi/CR4 interaction is not working in pauci-immune CrGN because there is no deposition of immunoglobulins or complements. Therefore, these results suggest that CR3 and CR4 might function as fibrin/fibrinogen receptors in extra-capillary lesions.

On the other hand, in immune-mediated

type of human CrGN, the expression of ICAM-1 was significantly correlated with the infiltration of beta2 integrin+ leukocytes only in intra-capillary areas but not in extra-capillary areas. Moreover, the deposition of FRA was not correlated with infiltration of beta2 integrin+ leukocytes either in intra- or extra-capillary areas. These findings suggest that ICAM-1/LFA-1, ICAM-1/CR3, FRA/CR3 or FRA/CR4 interactions are not involved in the glomerular infiltration of leukocytes in immune-mediated human CrGN, except for ICAM-1/LFA-1 interaction in intra-capillary infiltration of leukocytes.

It has recently reported that glomerular injury could be prevented by antibodies to ICAM-1 or LFA-1[20)-23)] or by fibrinolytic therapy in experimental CrGN[30)]. These findings indicate the therapeutic efficacy of the intervention of the interaction of ICAM-1 or fibrin/fibrinogen with their receptors, beta2 integrins, on glomerular leukocytes. Therefore, our current results suggesting differential functions and roles of beta2 integrins will be important in the selection of therapeutic approaches in the patients with CrGN.

Acknowledgments

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