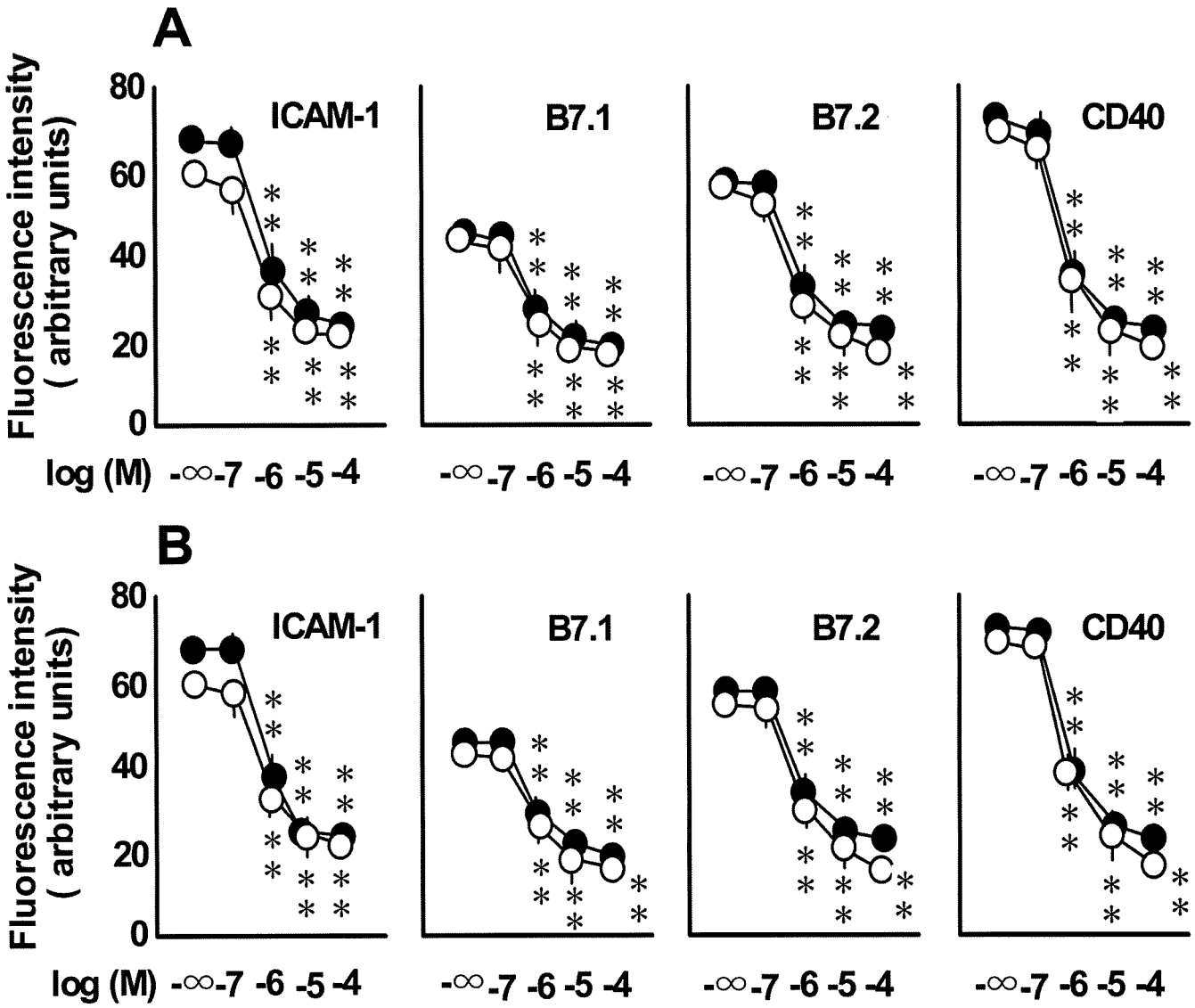

Fig. 11. The effects of forskolin and dbcAMP on AGE-induced ICAM-1, B7.1, B7.2, and CD40 expressions on human monocytes. PBMC at 1×10^6/ml were incubated with an adenylate cyclase activator, forskolin (A), and a cAMP analog, dbcAMP (B), at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 (filled circles; ●) and AGE-3 (open circles; ○) at 100 μg/ml for 24 h. The expressions of ICAM-1, B7.1, B7.2, and CD40 on monocytes were determined by flow cytometry. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

ulated with lipopolysaccharide, zymosan, or polymerized bovine albumin (Penglis et al., 2000), the expression of COX-2 was specifically up-regulated, leading to enhanced production of PGE_2. Lipopolysaccharide-treated monocytes/macrophages activate multiple signal transduction pathways, including the activation of NF-κB and c-Jun NH_2-terminal kinase. Some of these pathways, in part, may be shared by RAGE signaling. However, we confirmed that AGE-2, AGE-3, AGE-4, and AGE-5 at 100 μg/ml had no effect on the expression of COX-2 mRNA and protein in human monocytes (data not shown). In the present study, we examined the effect of a nonselective COX-2 inhibitor, indomethacin, and a selective COX-2 inhibitor, NS-398, on the actions of PGE_2 in the presence or absence of AGE-2 and AGE-3. The COX-2 inhibitors had no effect on the expressions of adhesion molecule, the cytokine production, and the lymphocyte proliferation (data not shown). In addition, AGE-2, AGE-3, AGE-4, and AGE-5 had no effect on PGE_2 production (data not shown). Therefore, it is probably that the endogenous production of PGE_2 in monocytes did not occur under the present conditions.

It is reported that PGE_2 induced by monocytes inhibits procollagen secretion by human vascular smooth muscle cells, leading to extracellular matrix remodeling and resistance to rupture during atherosclerosis (Fitzsimmons et al., 1999). Elevation of cAMP in endothelial cells inhibits proliferation, leading to the inhibition of atherosclerosis in patients with diabetes (Lorenowicz et al., 2007). Together with these results and our data, other extracellular stimuli, which induce intracellular cAMP production on binding to their cognate G protein-coupled receptors, may regulate the activation of vascular smooth muscle cells and endothelial cells. In conclusion, PGE_2 inhibited AGE-2-

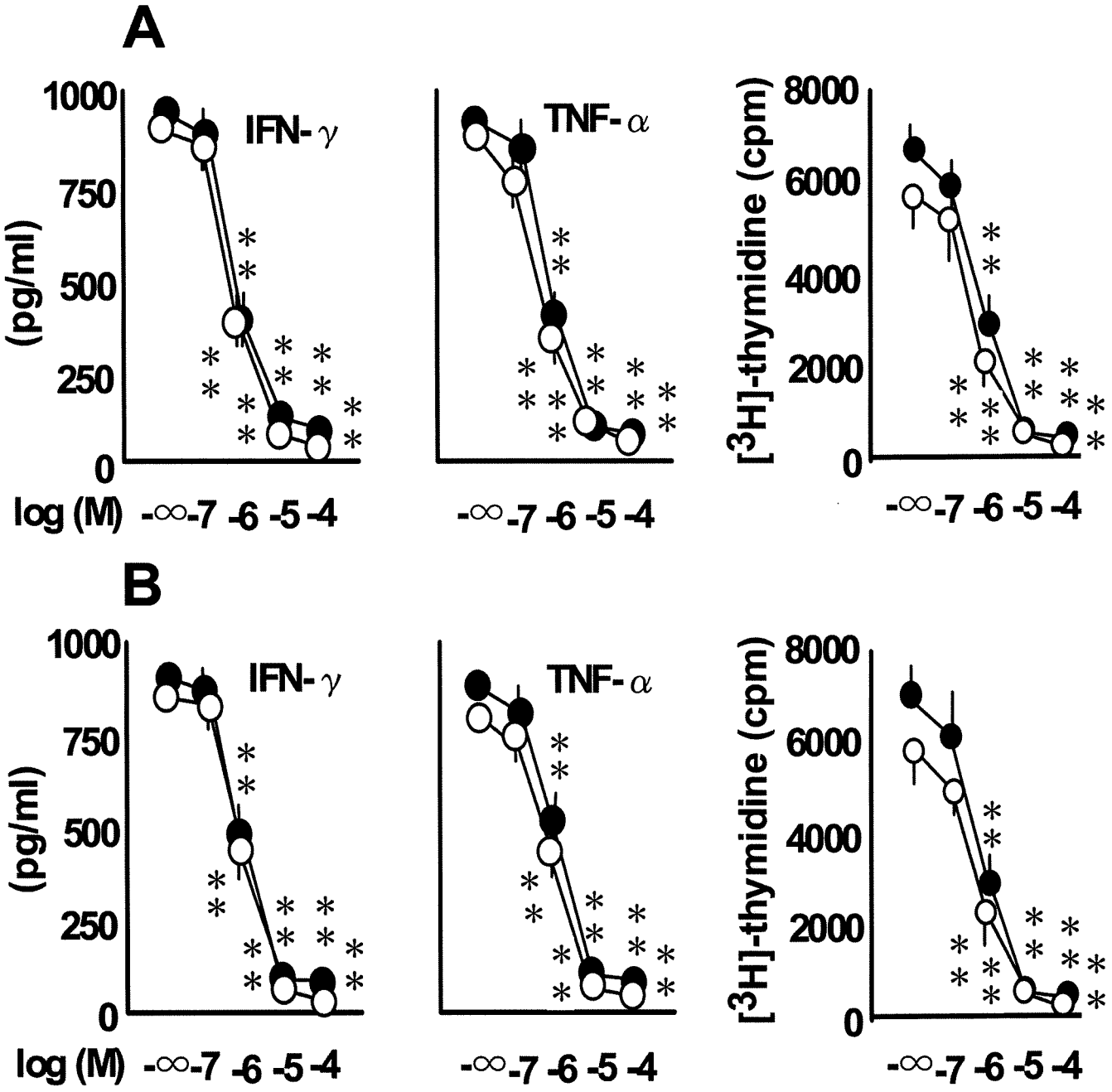

Fig. 12. The effects of forskolin and dbcAMP on AGE-induced IFN-γ and TNF-α production and the lymphocyte proliferation in PBMC. PBMCs at 1×10^6/ml were incubated with an adenylate cyclase activator, forskolin (A), and a cAMP analog, dbcAMP (B), at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 (filled circles; ●) and AGE-3 (open circles; ○) at 100 μg/ml for 24 h. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [^{3}H]thymidine uptake as described under *Materials and Methods*. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **, $P < 0.01$ compared with the values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

and AGE-3-induced expressions of ICAM-1, B7.1, B7.2, and CD40, the production of IFN-γ and TNF-α, and the lymphocyte proliferation via EP_2/EP_4 receptors and the cAMP/PKA pathway. Through the inhibition of toxic AGE-dependent responses in monocytes, the stimulation of EP_2 and EP_4 receptors may partially contribute to regulation of the development of atherosclerotic plaques in diabetes. The present study might lead to an exploration of the therapeutic potential of PGE_2 on the systemic inflammatory response evoked by diabetes.

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Original Article

High Mobility Group Box 1 Complexed with Heparin Induced Angiogenesis in a Matrigel Plug Assay

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Angiogenesis involves complex processes mediated by several factors and is associated with inflammation and wound healing. High mobility group box 1 (HMGB1) is released from necrotic cells as well as macrophages and plays proinflammatory roles. In the present study, we examined whether HMGB1 would exhibit angiogenic activity in a matrigel plug assay in mice. HMGB1 in combination with heparin strongly induced angiogenesis, whereas neither HMGB1 nor heparin alone showed such angiogenic activity. The heparin-dependent induction of angiogenesis by HMGB1 was accompanied by increases in the expression of tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor-A_{120} (VEGF-A_{120}). It is likely that the dependence of the angiogenic activity of HMGB1 on heparin was due to the efficiency of the diffusion of the HMGB1-heparin complex from matrigel to the surrounding areas. VEGF-A_{165} possessing a heparin-binding domain showed a pattern of heparin-dependent angiogenic activity similar to that of HMGB1. The presence of heparin also inhibited the degradation of HMGB1 by plasmin *in vitro*. Taken together, these results suggested that HMGB1 in complex with heparin possesses remarkable angiogenic activity, probably through the induction of TNF-α and VEGF-A_{120}.

Key words: angiogenesis, HMGB1, heparin

Angiogenesis is an essential process by which new blood vessels are formed and accompanies embryonic development, inflammation and wound healing. Angiogenesis may also be involved in several disease conditions, including cancers, rheumatic arthritis, diabetic retinopathy and atherosclerosis. The process of angiogenesis appears to be under the control of a complex system consisting of proangiogenic and antiangiogenic factors. From the cellular perspective, angiogenesis is a series of processes that induce the proliferation and migration of vascular endothelial cells (ECs), in association with the activation of macrophages. These cells together with stromal cells produce active substances such as matrix metalloproteinases (MMPs), a variety of cytokines and growth factors [1-3].

High mobility group box 1 (HMGB1), a non-histone nuclear protein involved in maintaining the architectural structure of DNA and the regulation of transcription, is secreted from necrotic cells and activated macrophages. Once released into the extracellular space, HMGB1 acts as a cytokine [4-8]. HMGB1 is

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widely distributed in all types of cells and is highly conserved throughout species [9]. HMGB1 has a unique structure consisting of 3 domains. The A-box and B-box domains are rich in basic amino acids and bind to DNA, while the C-terminal domain is composed of acidic amino acid clusters [10]. It has been suggested that amino acids 6–12 bind to heparin, and that amino acids 150–183 bind to the receptor for advanced glycation end products (RAGE) [11, 12]. The expression of HMGB1 is increased in inflammatory conditions such as sepsis [13], atherosclerosis [14] and rheumatic arthritis [15]. HMGB1 is also secreted from several tumor cells [16–20]. Secreted HMGB1 may bind to RAGE, toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) expressed on the surfaces of monocytes/macrohpages, and such bindings activate NF-κB [21–23], thereby facilitating the inflammatory response through the expression and secretion of inflammatory cytokines (tumor necrosis factor-α (TNF-α), IL-1α, IL-1β, IL-6, IL-8, *etc.*) [24]. In addition, HMGB1 may be closely related to angiogenesis during inflammation, since recent studies revealed that HMGB1 induces sprouting and chemotaxis of ECs and promotes angiogenesis in the chick embryo chorioallantoic membrane [25, 26]. However, there have been few studies on the effects of HMGB1 on angiogenesis in *in vivo* mammalian models.

In this study, we examined the effect of HMGB1 on angiogenesis *in vivo* using a matrigel system and demonstrated that HMGB1 induced angiogenesis in a heparin-dependent manner. This angiogenesis may occur through the expression of TNF-α and vascular endothelial growth factor-A_{120} (VEGF-A_{120}) in the surrounding areas of the matrigel.

Materials and Methods

Reagents. Matrigel is a mixture of basement membrane components extracted from Engelbreth-Holm-Swarm (EHS) tumors inoculated in C57BL/6J mice. The matrigel was prepared as previously described [27], and was not supplemented with any growth factors. Matrigel mainly contains laminin, collagen IV, heparan sulfate proteoglycan and entactin. Since matrigel is a liquid at 4℃ and turns into a gel at 22–35℃, we injected ice-cold matrigel solution into the backs of mice to allow the gel to form subcutaneously, as shown in Fig. 1. Anti-rat IgG goat polyclonal IgG-HRP was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rat monoclonal anti-bovine HMGB1 antibody (#10–22) was produced as described previously [28].

Animals. Male C57BL/6J mice (24–26 g, 7–8 wk) were obtained from the Department of Animal Resources of Okayama University. All animal experiments were performed according to the guidelines of Okayama University on animal experiments, were approved by the University's committee on animal experimentation and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Mice were housed with a 12-h light-dark cycle at 22℃ with water and food ad libitum.

Methods.

1. Expression and purification of HMGB1.

Full-length human HMGB1 DNA was amplified by PCR using Cap Site cDNA dT from human microvascular endothelial cells (Nippon Gene, Tokyo, Japan) and primers (forward 5'-GCA GAA TTC ATG GGC AAA GGA GAT CCT A-3', reverse 5'-CAT CTC GAG TCA TTA TTC ATC ATC ATC ATC-3'). The full-length HMGB1 DNA fragment and pGEX-6p-1 vector (GE Healthcare, Little Chalfont, England) were digested with EcoRI and XhoI (New England Biolabs, Ipswich, UK) restriction enzymes. The DNA fragment was subcloned into the vector. The recombinant plasmids were transformed into *E. coli* strain BL21 (DE3) (Merck, San Diego, CA, USA). The transformants were incubated overnight at 37℃ to express the recombinant GST-HMGB1 in Overnight Express Instant TB medium (Merck). *E. coli* extract containing GST-HMGB1 fusion proteins was applied to glutathione-Sepharose 4B and incubated for 1 h at room temperature. After extensive washing, the gel bed was incubated with PreScission Protease for 3 h at 4℃. After brief centrifugation, the supernatant containing GST-tag-deleted HMGB1 was collected and purified by gel filtration chromatography using TSK-gel 3000SW_{XL} (Tosoh, Tokyo, Japan) [29]. Purified recombinant human HMGB1 protein was identified by SDS-PAGE [30] and Western blotting [31] with anti-HMGB1 monoclonal antibody (#10–22). The final HMGB1 preparation contained lipopolysaccharide (LPS) of less than 2.0 pg/μg protein.

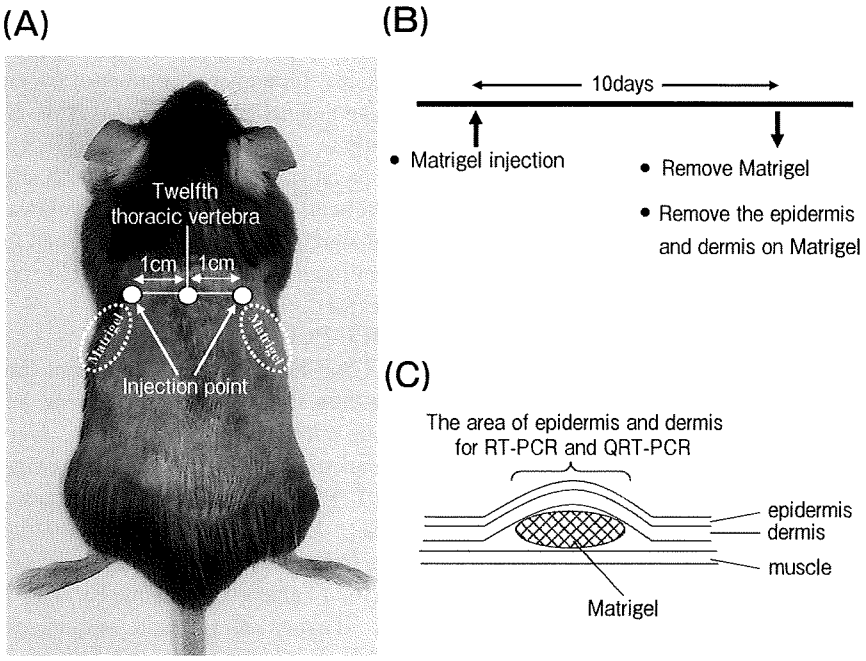

Fig. 1 *In vivo* matrigel angiogenesis plug assay method. (A) The injection point of matrigel into the mouse back is indicated. For the PBS control, matrigel was inoculated on the left side and for the other groups, it was inoculated on the right side. (B) The protocols of matrigel injection and sampling are shown. (C) The sampling area of mouse skin for RT-PCR or real-time PCR is shown.

2. *In vivo* matrigel plug assay.

At 4℃, 500μl of matrigel solution (8.4mg protein/ml) was mixed with PBS, HMGB1 (2.5μg/ml, 100nM), heparin (320μg/ml, 64units/ml) (Sigma, St. Louis, MO, USA), heparin + HMGB1, human VEGF-A_{121} (185ng/ml, 6.5nM or 1.85μg/ml, 65nM) (Peprotech, Rocky Hill, CT, USA), human VEGF-A_{165} (250ng/ml, 6.5nM) (Peprotech), human VEGF-A_{121} + heparin, or human VEGF-A_{165} + heparin. Male C57BL/6J mice were anesthetized with 50% N_2O + 50% O_2 and the liquid matrigel mixture was injected subcutaneously into the back using a 25G needle. The liquid matrigel mixture was solidified 5–10min after injection (Fig. 1A). Mice were sacrificed at the indicated time points by cervical dislocation and the matrigel plugs were recovered for analysis of angiogenesis (Fig. 1B).

3. Quantification of hemoglobin in matrigel.

The removed matrigel plugs were homogenized in 10mM PBS (pH7.4) and centrifuged at 8,000 × g for 10min. The hemoglobin contents in the supernatant were determined using a Hemoglobin B test kit (Wako, Osaka, Japan) according to the manufacturer's instructions.

4. Immunohistochemistry.

Matrigel plugs were fixed with 10% formalin-0.1 M phosphate buffer and embedded in paraffin for HE and immunohistochemical stainings. Immunohistochemical staining of CD31 was performed on 5μm matrigel sections with rabbit polyclonal anti-mouse CD31 antibody (Abcam, Cambridge, UK) followed by a secondary antibody, anti-rabbit IgG goat IgG-HRP (Abcam). Immunoreactivity was visualized with 0.05% 3, 3'-diaminobenzidine (Sigma) and 0.03% H_2O_2. Counterstaining of nuclei was performed with Mayer's hematoxylin. A negative control for immunohistochemical staining was established by using the same concentration of normal rabbit IgG. CD31-positive vessels were counted in 5 fields of a matrigel plug section at 400 × magnification under a microscope. Microvessel density (MVD) was expressed as the number of microvessels per square millimeter.

5. HMGB1-heparin binding assay.

Heparin-Sepharose CL-6B (GE Healthcare) equilibrated with PBS (10μl gel bed) was added to 200μl of HMGB1 (400μg/ml in PBS) solution and incubated

for 16h at 4℃ with gentle shaking. The reaction mixture was centrifuged at 8,000×g for 10min at 4℃. The supernatant was collected, and the Sepharose was washed with PBS three times. The supernatant and the resultant Sepharose gel samples were analyzed by SDS-PAGE to examine the binding of HMGB1 to heparin.

6. HMGB1 digestion assay.

Ten μl of HMGB1 (500μg/ml in PBS) was mixed with 2.5μl of heparin (32 or 320μg/ml) or PBS and incubated for 16h at 4℃. Additionally, the mixtures were mixed with 2.5μl of plasmin (20μg/ml) (Merck) and incubated for 1h at 25℃. The reaction mixtures were analyzed by SDS-PAGE.

7. Determination of HMGB1 levels in matrigel using Western blotting.

A matrigel mixture containing PBS, HMGB1 (2.5μg/ml, 100nM) or heparin (320μg/ml, 64units/ml) + HMGB1 was injected subcutaneously into the backs of mice. After 1, 3 or 10 days, the matrigel plugs were recovered. Matrigel homogenate corresponding to 2.5mg wet weight was electrophoresed on polyacrylamide gel (12.5%). The samples were electrophoretically blotted on polyvinylidine difloride membrane (Bio-rad, Hercules, CA, USA) at 45 V for 2h by a transblot apparatus. After the membrane was stained by ponceau S, it was incubated with 10% skim milk for 1h and was incubated overnight at 4℃ with mouse monoclonal anti-human HMGB1 antibody (R&D Systems, Minneapolis, MN, USA). After washing, the membrane was incubated with anti-mouse IgG goat polyclonal IgG-HRP (MBL, Nagoya, Japan) for 1h. The signals were finally visualized using an enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL, USA).

8. RT-PCR.

Total RNA was isolated from the mouse skin surrounding the matrigel plug using an RNeasy fibrous tissue mini kit (Qiagen, Hilden, Germany) (Fig. 1C). Complementary DNA was synthesized with a Takara RNA PCR kit Ver. 3.0 (Takara Bio, Nagahama, Japan) according to the manufacturer's instructions. The PCR reaction was performed in a PCR Thermal Cycler (Takara Bio) using Ex Taq DNA polymerase HS (Takara Bio), and sequence-specific primers (Table 1) according to the following program: after the initial denaturation at 94℃ for 3min, amplification was done using X cycles of 94℃ for 30 sec, Y℃ for 30 sec, and 72℃ for 60 sec, followed by a final extension at 72℃ for 7min (X and Y are defined in

Table 1 Primer sequences, annealing temperatures, amplification cycles and product sizes for RT-PCR

Primer		Sequence 5'-3'	Amplification cycles (X)	Annealing temperature (Y)	Product size
GAPDH	forward	ACCACAGTCCATGCCATCAC	27	55°C	452bp
	reverse	TCCACCACCCTGTTGCTGTA			
TNF-α	forward	TTCTGTCTACTGAACTTCGG	37	55°C	354bp
	reverse	GTATGAGATAGCAAATCGG			
iNOS	forward	GCTTAGAGAACTCAACCAC	37	55°C	454bp
	reverse	GCTGCCCTCGAAGGTGAGC			
eNOS	forward	CTGGACACCAGGACAACC	37	55°C	460bp
	reverse	GCTGCTGTGCGTAGCTCT			
MMP-2	forward	CCGTGGATGATGCTTTTGC	30	55°C	319bp
	reverse	CTGTATTCCCGACCGTTGA			
MMP-9	forward	TGCGCCACCACAGCCAAC	35	60°C	399bp
	reverse	GCCACGACCATACAGATAC			
VEGFR-1	forward	TGTGGAGAAACTTGGTGACCT	35	55°C	505bp
	reverse	TGGAGAACAGCAGGACTCCTT			
VEGFR-2	forward	AAGTGATTGAGGCAGACGCT	37	55°C	524bp
	reverse	TGATGCCAAGAACTCCAT			
bFGF	forward	GACCCCAAGCGGCTCTACTGC	35	55°C	298bp
	reverse	GTGCCACATACCAACTGGAGT			
VEGF-A	forward	GCGGGCTGCCTCGCAGT	37	55°C	VEGF-A_{120} 275bp
	reverse	TCACCGCCTTGGCTTGTCA			VEGF-A_{164} 407bp
					VEGF-A_{188} 479bp

Table 1). The PCR product was analyzed by electrophoresis on 2% agarose gel.

9. Real-time quantitative PCR.

Real-time PCR was performed with a Light Cycler (Roche, Basel, Switzerland) according to the manufacturer's instructions. Reaction mixtures contained cDNA template, SYBR premix Ex Taq (Takara Bio, Shiga, Japan), and sequence-specific primers (Table 2).

10. Statistical analysis.

All data are presented as the means ± SEM. Differences between 2 groups were determined by ANOVA followed by Student's *t*-test. P values < 0.05 were considered statistically significant.

Results

The effect of HMGB1 and heparin on angiogenesis in a matrigel plug assay. The matrigel mixture was injected subcutaneously into the backs of mice as shown in Fig. 1. After 10 days, the matrigel plugs were recovered as shown in Fig. 2. It has been reported that 50 nuclei in the muscle tissue contain ~ 25 pg HMGB1 [32] and that LPS (100 ng/ml)-stimulated RAW264.7 cells (5×10^6 cells) release 10 μg HMGB1 into the culture medium [6]. Given that an individual cell has a volume of 10 μm^3 and 10% of the reported HMGB1 values represent extracellular release, the estimated concentration of HMGB1 in the tissues will be 0.05 (muscle tissue) and 0.2 (RAW264.7 cells) mg/ml, respectively. The concentrations of HMGB1 used in the present matrigel assay (2.5 μg/ml, 100 nM) corresponded to 5% and 1.25% of these estimated values, which would be readily attainable percentages under *in vivo* conditions in an inflammation area. The concentration of heparin (320 μg/ml, 64 units/ml) was determined according to the reports of Passaniti *et al.* [33] and Isaji *et al.* [34], who used FGF and VEGF as angiogenic growth factors, respectively. In the present study, angiogenesis did not appear to be induced in the phosphate-buffered saline (PBS) control group. Also in the groups treated with HMGB1 alone, the matrigel did not show any change compared with the PBS control. In the

Table 2 Primer sequences, annealing temperatures and product sizes for real-time PCR

Primer		Sequence 5'-3'	Annealing temperature	Product size
GAPDH	forward	TGACGTGCCGCCTGGAGAAA	55°C	98 bp
	reverse	AGTGTAGCCCAAGATGCCCTTCAG		
TNF-α	forward	GACCCTCACACTCAGATCATCCTTCT	55°C	106 bp
	reverse	GCGCTGGCTCAGCCACTC		
iNOS	forward	GGCAGCCTGTGAGACCTTTG	55°C	72 bp
	reverse	GCATTGGAAGTGAAGCGTTTC		
eNOS	forward	CAACGCTACCACGAGGACATT	55°C	90 bp
	reverse	CTCCTGCAAAGAAAAGCTCTGG		
MMP-2	forward	CCCCGATGCTGATACTGA	55°C	152 bp
	reverse	CTGTCCGCCAAATAAACC		
MMP-9	forward	GCCCTGGAACTCACACGACA	55°C	85 bp
	reverse	TTGGAAACTCACACGCCAGAAG		
VEGFR-1	forward	GGCAGACCAATACAATCCTAGATG	55°C	140 bp
	reverse	ACCAGGGTAATTCCAGCTCATTT		
VEGFR-2	forward	CGACATAGCCTCCACTGTTTATG	55°C	109 bp
	reverse	TTTGTTCTTGTTCTCGGTGATGT		
bFGF	forward	CCCACCAGGCCACTTCAA	55°C	71 bp
	reverse	GATGGATGCGCAGGAAGAA		
VEGF-A_{120}	forward	GCCAGCACATAGAGAGAATGAGC	55°C	104 bp
	reverse	CGGCTTGTCACATTTTTCTGG		
VEGF-A_{164}	forward	GCCAGCACATAGAGAGAATGAGC	55°C	97 bp
	reverse	CAAGGCTCACAGTGATTTTCTGG		
VEGF-A_{188}	forward	GCCAGCACATAGAGAGAATGAGC	55°C	171 bp
	reverse	AACAAGGCTCACAGTGAACGCT		

heparin-treated group, there was a low level of angiogenesis, judging from the macroscopic color change. In the group treated with the combination of HMGB1 and heparin, the whole gel had red color heavily, and a lot of the blood vessels seemed to be elongated into the center of the matrigel (Fig. 2A). Next, we determined the hemoglobin contents in the matrigel isolated from the back. We obtained consistent results that reflected the color changes described above. In the PBS or HMGB1 group, little hemoglobin was detected in the matrigel. In the heparin group, there was a small amount of hemoglobin detected in the gel. In the group treated with the combination of HMGB1 and heparin, the hemoglobin content was significantly increased (Fig. 2B). Taken together, these results indicate that angiogenesis was induced by the combination of HMGB1 and heparin in the matrigel plug assay. Ten days after inoculation, a thin section of the matrigel was stained with hematoxylin-eosin (HE) (Fig. 3). In the group treated with the combination of HMGB1 and heparin, many infiltrating cells, including leukocytes, were observed in the gel (Fig. 3A, panel d). However, few cells were observed in the gel sections from other groups (Fig. 3A, panels a-c). Secondly, immunostaining was performed with an antibody against CD31 that is a marker antigen of vascular endothelial cells. Many cells with strong positively for CD31 were seen to be forming luminal structures in the sections from matrigels treated with the combination of HMGB1 and heparin (Fig. 3A, panel h). The staining with anti-CD31 antibody was specific, because staining with the control antibody did not reveal any structures in the same section. In other groups, few CD31-positive structures were observed (Fig. 3A, panels e-g). The number of CD31-positive luminal structures was counted in five random fields. In the group treated with HMGB1 and heparin, the number of CD31-positive luminal structures was significantly increased in comparison with the number by treatment with HMGB1 or heparin alone (Fig. 3B). Taken together, these results showed that the combination of HMGB1 and heparin induced the migration of vascular endothelial cells, leading to the formation of new vessels in the matrigel.

HMGB1 binds to heparin. SDS-PAGE analysis following the incubation of HMGB1 with heparin-Sepharose gel revealed that HMGB1 was present in the heparin-Sepharose gel fraction but not in the supernatant fraction, indicating that HMGB1 formed a complex with heparin (Fig. 4A).

Degradation of HMGB1 by plasmin in the presence or absence of heparin. It has been reported that HMGB1 is a good substrate for plasmin [35]. Therefore, we examined whether the complex formation between HMGB1 and heparin affected the digestion efficiency by plasmin. As shown in Fig. 4B, HMGB1 was resistant to plasmin digestion in the presence of heparin. Even at a lower concentration (32μg/ml: 1/10 the concentration of heparin used in the matrigel assay), the presence of heparin inhibited the degradation of HMGB1 by plasmin.

The determination of HMGB1 levels in matrigel. A matrigel mixture containing PBS, HMGB1 or heparin + HMGB1 was injected subcutaneously into the backs of mice. After 1, 3 or 10 days, the mice were sacrificed and the matrigel plugs were recovered. HMGB1 was detected by Western blotting with anti-HMGB1 monoclonal antibody. An equivalent amount of matrigel homogenate, corresponding to 2.5 mg wet weight, was loaded on each lane (Fig. 5A). In the group treated with HMGB1 and heparin, HMGB1 disappeared completely from the matrigels 3 days after inoculation, whereas there was no marked change in HMGB1 level in the group treated with HMGB1 alone throughout the 10-day measurement period (Fig. 5B).

The expression of angiogenesis-related mRNA in the mouse skin surrounding the matrigel plug. Total RNA was extracted from mouse skin surrounding the matrigel plug. Ten days after the inoculation of matrigel, RT-PCR for TNF-α, inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), MMP-2, MMP-9, VEGF receptor-1 (VEGFR-1), VEGFR-2, basic fibroblast growth factor (bFGF) and VEGF-A was performed with extracted RNA, and the band density of each group was compared after agarose gel electrophoresis. The TNF-α mRNA level was higher in the HMGB1 group than the PBS group. The TNF-α mRNA level was significantly higher in the group treated with HMGB1 and heparin than in that treated with HMGB1 alone. The expression of TNF-α mRNA in the other groups was minimal under the same PCR conditions. There were no significant differences in iNOS, eNOS, MMP-2, MMP-9, VEGFR-1, VEGFR-2 or bFGF mRNA levels among the groups.

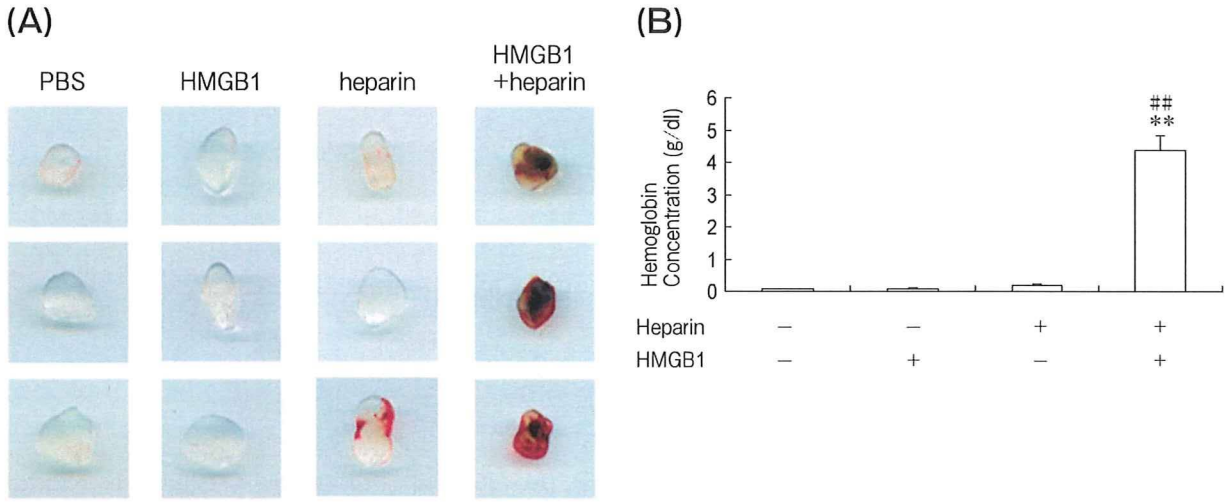

Fig. 2 Effect of HMGB1 and heparin on angiogenesis in the matrigel assay. (A) A matrigel mixture containing HMGB1 and heparin alone or in combination was injected subcutaneously into the backs of mice. After 10 days, the matrigel plugs were recovered. Each group consisted of 6 mice. Three representative cases are shown. (B) The concentrations of hemoglobin in the matrigel plugs were determined. The results are the means ± SEM of 6 matrigels. $^{**}P<0.01$ compared with the PBS control group without any treatment. $^{\#\#}P<0.01$ compared with the group treated with heparin alone.

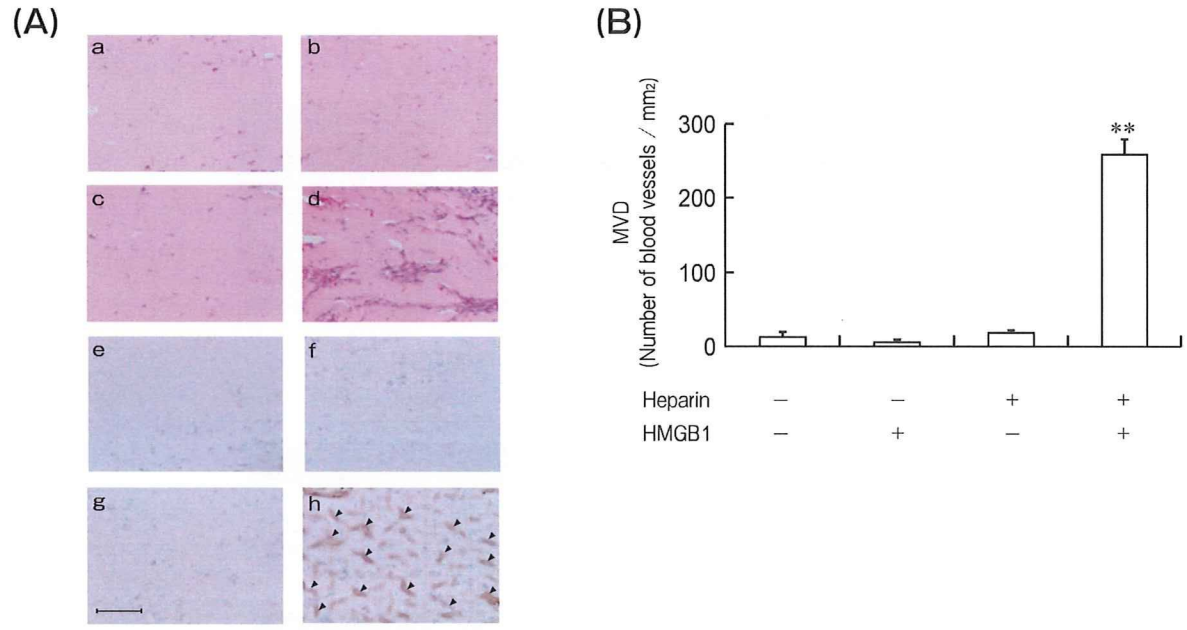

Fig. 3 Histological study of matrigel plugs. (A) The matrigels were removed from the mouse back 10 days after inoculation and fixed in 10% formalin. Paraffin-embedded sections of matrigel plug were stained by hematoxylin-eosin (a-d) and by anti-CD31 antibody (e-h). The matrigels contained PBS (a, e), HMGB1 alone (b, f), heparin alone (c, g), or a combination of HMGB1 and heparin (d, h). Arrowheads indicate CD31-positive blood vessels. The scale bar represents 100μm. (B) CD31-positive vessels were counted in 5 fields of the matrigel plug section at 400 × magnification under a microscope. The microvessel density (MVD) was expressed as the number of microvessels per square millimeter. The results are the means ± SEM of 5 fields. $^{**}P<0.01$ compared with the PBS control group.

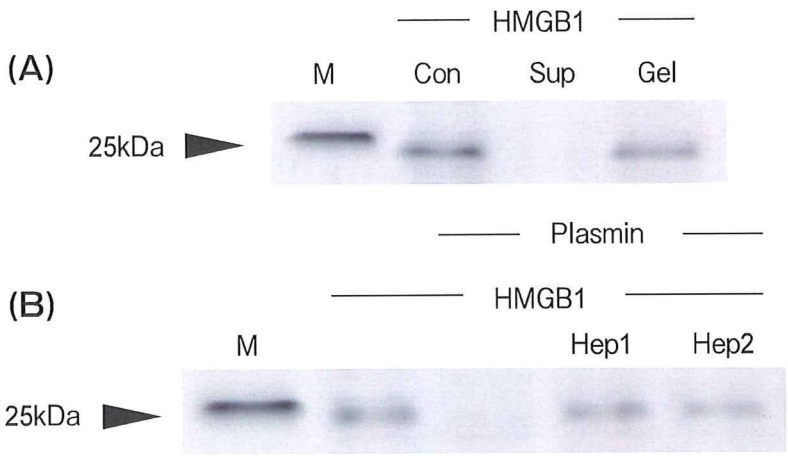

Fig. 4 HMGB1 binding to heparin and its effect on digestion by plasmin. (A) HMGB1 was mixed with heparin-Sepharose and incubated for 16h at 4°C. After incubation, the supernatant (Sup) and the protein from the heparin-Sepharose gel (Gel) were electrophoresed on 12% SDS-PAGE gel under a reducing condition, followed by staining with Coomassie-blue. (M) is a molecular weight marker. (Con) represents the HMGB1 control. (B) HMGB1 was preincubated with or without heparin for 16h at 4°C. After preincubation, the mixtures were subjected to plasmin digestion for 1h at 25°C. The resultant mixtures were electrophoresed and the gels were stained as mentioned above. (Hep1) is HMGB1 incubated with 32μg/ml heparin and plasmin. (Hep2) is HMGB1 incubated with 320μg/ml heparin and plasmin.

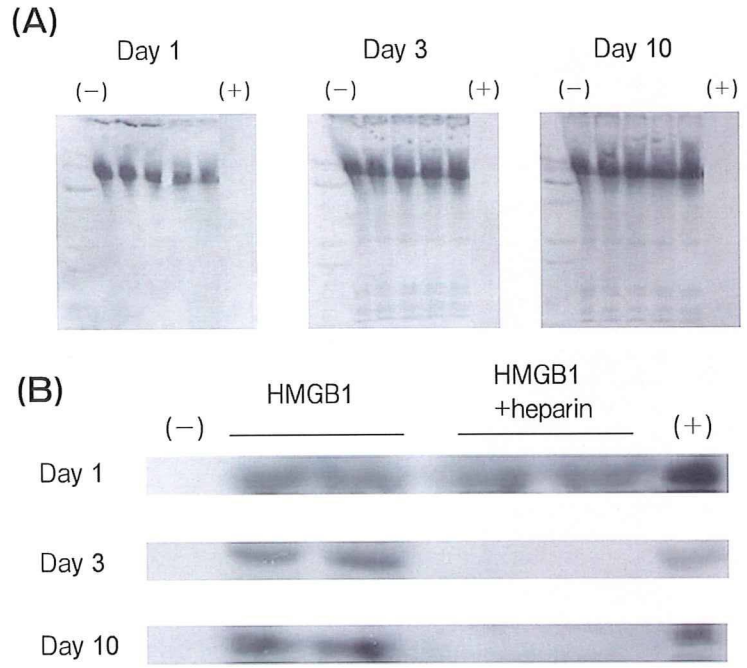

Fig. 5 Determination of HMGB1 levels in matrigel using Western blotting. A Matrigel mixture containing PBS, HMGB1 (2.5μg/ml, 100nM) or heparin (320μg/ml, 64units/ml) + HMGB1 was injected subcutaneously into the backs of mice. After 1, 3 and 10 days, the matrigel plugs were recovered. The matrigel plugs were homogenized and an amount of homogenate corresponding to 2.5mg wet weight of matrigel was loaded on each lane. (A) Ponseau S staining of the PVDF membrane. (B) Detection of HMGB1 in matrigel. (−) negative control; matrigel contains PBS alone. (+) positive control; recombinant human HMGB1.

We also examined the mRNA levels of 3 kinds of VEGF isoform (VEGF-A_{120}, VEGF-A_{164}, VEGF-A_{188}). There were no significant differences in the mRNA levels of VEGF-A_{164} or VEGF-A_{188} among the groups. However, the VEGF-A_{120} mRNA expression level was significantly higher in the group treated with HMGB1 and heparin than in the PBS control and heparin-alone groups (Fig. 6). Consequently, it was revealed that the combination of HMGB1 and heparin selectively increased the expressions of the mRNAs of TNF-α and VEGF-A_{120}.

Quantification of angiogenesis-related mRNA in mouse skin. The mRNA expression levels of cytokines, MMPs and growth factors were quantified by real-time PCR using the RNA extracted from mouse skin surrounding the matrigel plug (Figs. 7A and B). The expression of TNF-α mRNA was significantly higher in the group treated with HMGB1 alone than in that treated with PBS alone. In addition, the TNF-α mRNA expression was significantly higher in the group treated with the combination of HMGB1 and heparin than in that treated with HMGB1 alone (Fig. 7A). In regard to the expressions of iNOS, eNOS, MMP-2, MMP-9, VEGFR-1, VEGFR-2 and bFGF, there were no differences among the groups, in agreement with the RT-PCR findings. In regard to the VEGF-A mRNA expression level, VEGF-A_{120} mRNA was significantly higher in the group treated with the combination of HMGB1 and heparin than in the other groups, whereas the expression of mRNAs for VEGF-A_{164} and VEGF-A_{188} was not different among the groups (Fig. 7B). Collectively, these findings demonstrated that the combination treatment with HMGB1 and heparin increased the mRNA expression levels of TNF-α and VEGF-A_{120} significantly.

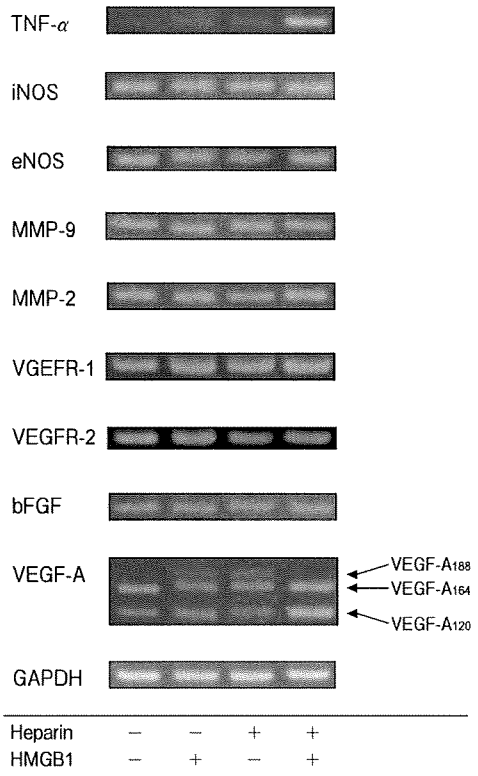

Fig. 6 Expression of mRNAs in mouse skin surrounding the matrigel plug. (A) Total RNA was isolated from the skin above the matrigel plug at 10 days after matrigel injection into the backs of mice. RT-PCR was performed using the primers indicated in Table 1. Representative results from 3 mice are shown.

The effect of VEGF and heparin on angiogenesis in the matrigel plug assay. It was apparent that the combination of VEGF-A_{165} (250 ng/ml, 6.5 nM) and heparin also induced angiogenesis 10 days after inoculation of matrigel (Fig. 8). The combination of VEGF-A_{121} (185 ng/ml, 6.5 nM) and heparin did not induce angiogenesis significantly. VEGF-A_{165} alone also did not induce angiogenesis. A tenfold higher concentration (1.85 μg/ml, 65 nM) of VEGF-A_{121} alone did not induce angiogenesis in the matrigel plug, but induced a low level of angiogenesis in the skin surrounding it (Fig. 9).

Discussion

In the present study, it was clearly demonstrated that the combination of HMGB1 and heparin induced marked angiogenesis in the matrigel plug assay, accompanied with many CD31-positive blood vessels in the gel, whereas HMGB1 or heparin alone did not. Since matrigel, the basement membrane components purified from murine EHS tumors, mainly contains laminin, collagen IV, heparan sulfate proteoglycan and entactin [27] and HMGB1 has been repeatedly reported to bind to heparin and heparan sulfate proteoglycan [36-38], HMGB1 is assumed to bind to heparan sulfate proteoglycan in the matrigel plug in the absence of heparin. For this reason, we expected that the diffusion of HMGB1 to the surrounding tissue would be suppressed and HMGB1 would fail to leave

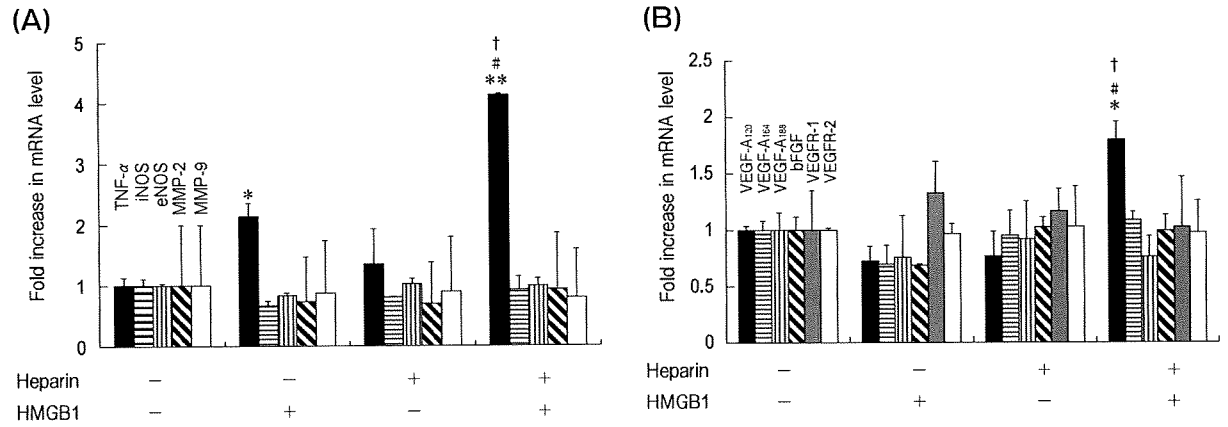

Fig. 7 Quantification of mRNA expression in mouse skin surrounding matrigel plug. Total RNA was isolated from mouse the skin above the matrigel plug at 10 days after matrigel injection into the backs of mice. Real-time PCR was performed using the primers indicated in Table 2. The expression of GAPDH was used to normalize cDNA levels. (A) The columns represent the following: ■, TNF-α; ▤, iNOS; ▥, eNOS; ▧, MMP-2; □, MMP-9. (B) The columns represent the followings; ■, VEGF-A_{120}; ▤, VEGF-A_{164}; ▥, VEGF-A_{188}; ▧, bFGF; ▩, VEGFR-1; □, VEGFR-2. The results are the means ± SEM for 3 animals. $^{*}P<0.05$, $^{**}P<0.01$ compared with the control group in the absence of HMGB1 and heparin. $^{\#}P<0.05$ compared with the group treated with HMGB1 alone. $^{\dagger}P<0.05$ compared with the group treated with heparin alone.

the matrigel and stimulate the tissue surrounding the matrigel plug. And in fact, this notion was supported by the results showing the presence of initial levels of HMGB1 in the matrigel up to 10 days after inoculation. In contrast, HMGB1 completely disappeared from the matrigel in the presence of heparin 3 days after inoculation. Thus, the addition of heparin to HMGB1 probably allows the HMGB1 to diffuse to the surrounding tissue through its complexation with heparin. The HMGB1-heparin complex probably does not bind to the heparan sulfate proteoglycan in matrigel or on cell surface, and thus it can bind to the receptors of HMGB1 (*e.g.*, RAGE, TLR-2 and TLR-4) [11] on the surface of macrophages and vascular endothelial cells. Since VEGF, a well-known angiogenic factor, has been reported to become resistant to degradation by proteinases upon complexation with heparin [1] and HMGB1 is known to be degraded by plasmin efficiently [35], we examined whether HMGB1 degradation by plasmin may be changed after the complex formation with heparin. As shown in Fig. 4B, the degradation of HMGB1 by plasmin was significantly retarded in the presence of heparin, suggesting the acquirement of resistance to enzymatic digestion. Together, these results show that complex formation with heparin enabled HMGB1 to acquire resistance to proteolytic degradation and to diffuse to the surrounding tissues, thus leading to angiogenenic activity.

We observed that HMGB1 in combination with heparin increased the expression of TNF-α and VEGF-A_{120} significantly. TNF-α has been reported to induce IL-8, VEGF and bFGF expression in human microvascular endothelial cells. Also, TNF-α facilitates tube formation in HMVEC *in vitro* and angiogenesis on chick chorioallantoic membranes and rat corneas *in vivo*. Thus, TNF-α may have a direct angiogenic action as well as an indirect action through IL-8, VEGF and bFGF [39-41]. In the present study, HMGB1 alone also induced the expression of TNF-α mRNA but did not induce angiogenesis. The TNF-α expression level induced by HMGB1 alone may not be sufficient to induce angiogenesis. However, the high expression level of TNF-α mRNA induced by the combination of HMGB1 and heparin is thought to be sufficient to induce angiogenesis. Moreover, in the matrigel plug assay, TNF-α has been reported to induce angiogenesis in a heparin-dependent manner [42]. Taken together, these results suggest that TNF-α induced by HMGB1 in complex with heparin may contribute to angiogenesis to some degree. Five kinds of isoforms of VEGF-A are presently known to exist: VEGF-A 120/121, 144/145, 164/165, 188/189, and 205/206 (murine/human). VEGF-$A_{120/121}$,

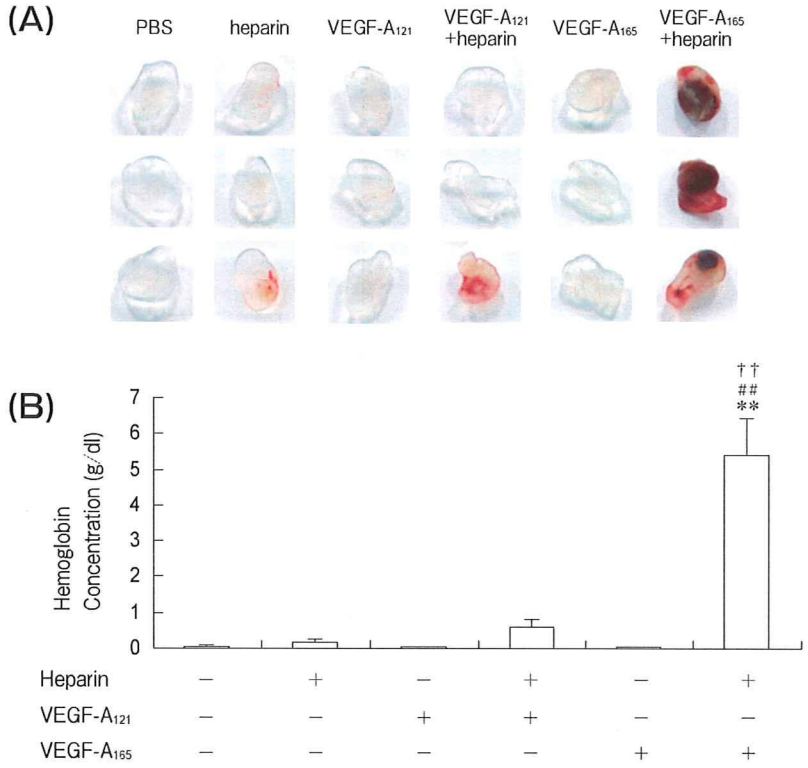

Fig. 8 Effect of VEGF on the angiogenesis in the presence or absence of heparin in the matrigel assay. (A) A matrigel mixture containing VEGF (VEGF-A_{121} 185 ng/ml, 6.5 nM; VEGF-A_{165} 250 ng/ml, 6.5 nM) and heparin alone or in combination was injected subcutaneously into the backs of mice. After 10 days, the matrigel plugs were recovered. Each group consisted of 6 mice. (B) The concentrations of hemoglobin in the matrigel plugs were determined. The results are the means ± SEM of 6 matrigels. $^{**}P<0.01$ compared with the PBS control group without any addition. $^{\#\#}P<0.01$ compared with the group treated with heparin alone. $^{\dagger\dagger}P<0.01$ compared with the group treated with VEGF-A_{165} alone.

VEGF-$A_{144/145}$ and VEGF-$A_{188/189}$ have been reported to be expressed widely in many kinds of tissue. Among these three isoforms, VEGF-$A_{120/121}$ and VEGF-$A_{164/165}$ are known to induce angiogenesis [3]. VEGF-$A_{120/121}$ is the only isoform lacking heparin-binding ability, because it does not have exon 6 and 7 [3]. Taken together, these findings suggest that HMGB1 in complex with heparin induces angiogenesis through the expression of TNF-α and VEGF-A_{120}.

Interestingly, VEGF-A_{165} with a heparin-binding domain showed a pattern of activities similar to HMGB1; that is, both showed heparin-dependent angiogenic activity. This implies that the heparin-binding of VEGF-A_{165} may also be important for diffusion of VEGF-A_{165} from matrigel and protection from proteolytic digestion [1] in this model. Although VEGF-A_{121} lacking heparin-binding ability did not show any angiogenic activity even in the presence of heparin, a high concentration of VEGF-A_{121} (1.85 μg/ml, 65 nM) induced a low level of angiogenesis in the skin surrounding the matrigel plug. The low angiogenic activity of VEGF-A_{121} might be ascribed to degradation by proteases during diffusion. Thus it is likely that the VEGF-A_{120} produced in the tissue surrounding the matrigel plug by the combination of HMGB1 and heparin works on vascular endothelial cells and macrophages in an autocrine and a paracrine manner at relatively higher concentrations. This may limit the proteolytic inactivation of VEGF-A_{120} induced by HMGB1 and heparin. Further studies will be needed to investigate this point.

In inflammation and tissue injury, HMGB1 is released from the nuclei of necrotic or damaged cells. The released HMGB1 functions as a proinflammatory

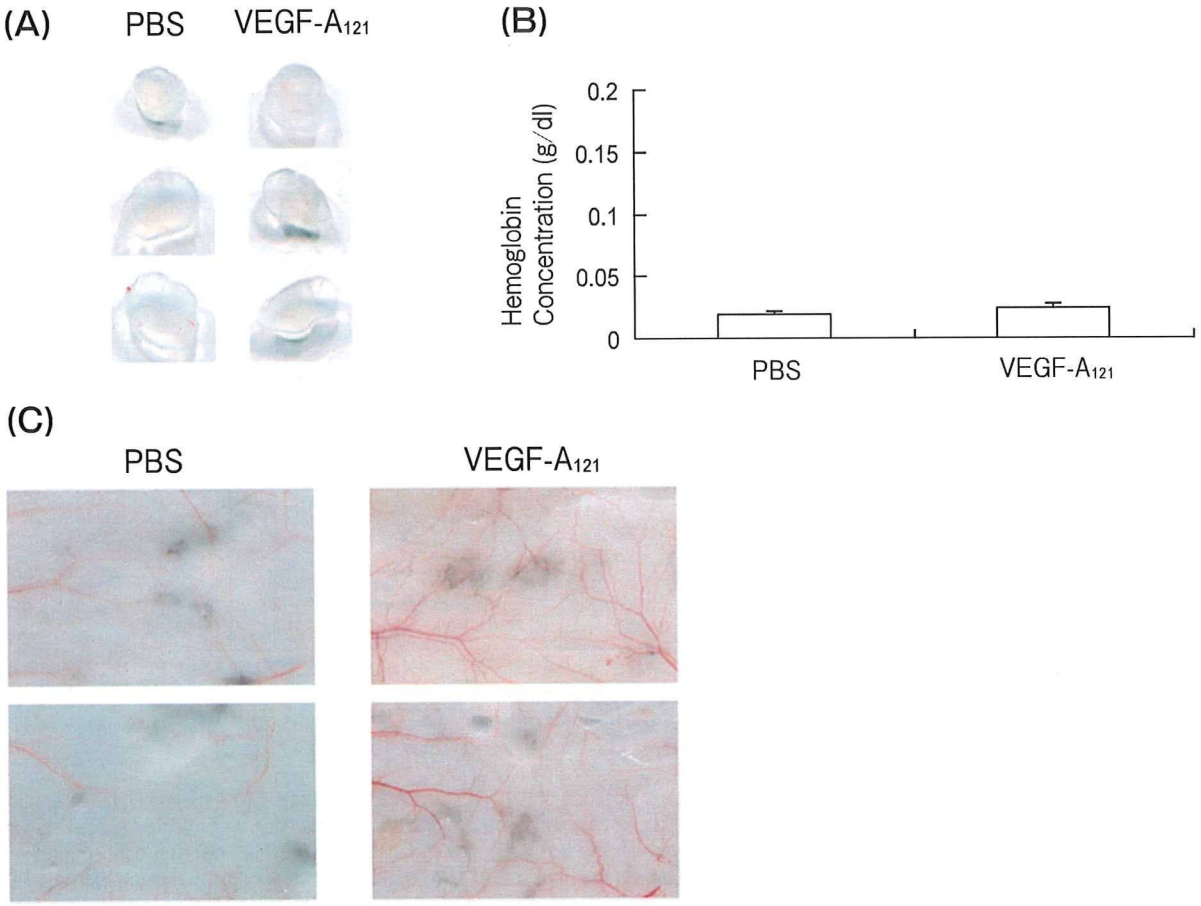

Fig. 9 Effect of high concentration VEGF-A_{121} alone on the angiogenesis. (A) A matrigel mixture containing a high concentration of VEGF-A_{121} (1.85 μg/ml, 65 nM) was injected subcutaneously into the backs of mice. After 10 days, the matrigel plugs were recovered. Each group consisted of 6 mice. (B) The concentrations of hemoglobin in the matrigel plugs were determined. The results are the means $\pm$ SEM of 6 matrigels. (C) A skin sample from the region surrounding the matrigel plug containing PBS or a high concentration of VEGF-A_{121} is shown.

cytokine through plural pathways [11]. However, HMGB1 trapped by heparan sulfate in the extracellular matrix cannot diffuse to surrounding areas. Mast cells secrete heparin [43], and this heparin can compete for heparan sulfate followed by the de-anchoring of HMGB1 from the extracellular matrix. On the other hand, macrophages and mast cells secrete heparanase [44, 45] and can degrade the heparan sulfate on which HMGB1 is anchored. HMGB1 bound to heparin or cleaved heparan sulfate can diffuse to larger surrounding areas and cannot be degraded easily by proteinase, allowing HMGB1 to bind to its receptors on macrophages and ECs. These events may play a role in tumor growth. It has been demonstrated that many types of cancer cells produce HMGB1 and release it extracellularly [16–20], and that mast cells and macrophages are abundant in the invasive front of tumors [46, 47]. Accumulating evidence indicates that VEGF production is involved in the angiogenesis of several cancers. Therefore, it is speculated that HMGB1 released from tumor cells may contribute to the angiogenic activity through the production of VEGF-A_{120} in the areas surrounding tumor tissues.

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—Foreword—

抗体医薬が切り拓く先端医療

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Advanced Targeting Therapy by Antibody Drugs

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現在，日本や欧米で20種以上の「抗体医薬」が認可を受け，今や抗体医薬の開発は創薬研究に不可欠なものとなってきている．難治性疾患に対する「抗体医薬」の著しい有効性は，抗体のヒト化技術の目覚ましい発展と重なり合って，21世紀の抗体医薬開発にとって大きなフロンティアが広がっていることを予感させる．また，ポストゲノムの時代となって次々と未知の遺伝子産物が抗原として用いられるようになったことは，抗体開発のスピードをより加速させている．すなわち，抗原性のハードルを乗り越えることのできるキメラ抗体やヒト化抗体の製造技術が確立されたこと，ゲノム科学の進歩によって医薬品開発の標的となり得る疾患関連因子の機能解明や探索技術が格段に発展してきたことが，今日における抗体医薬開発の隆盛をもたらしたと言える．このような背景の下，本シンポジウムにおいては，これまでに抗体医薬開発に携わってこられた研究者，臨床的立場から抗体医薬にかかわってこられた研究者，そして新たな抗体医薬の展開を試みる研究者に，それぞれの立場から抗体医薬の現状と展望について議論して頂き，今後の薬学領域における抗体医薬開発の方向性について考えていきたい．

まず，IgG型抗体のFc領域に結合している*N*型複合糖鎖からフコースを除去することによる抗体依存性細胞傷害活性（antibody-dependent cellular cytotoxity：ADCC活性）の増強作用の発見と抗腫瘍効果について論ずるとともに，増強メカニズムの解析，薬効評価，高ADCC活性型の低フコース抗体の製造技術（ポテリジェント技術）への応用を中心に紹介したい．

次に，抗体医薬のシーズとなり得る高特異性，高親和性の単クローン抗体を効率よく迅速に取得する技術を開発することは，抗体医薬を創薬して行く上で極めて重要なポイントであるが，動物を免疫する代わりに培養B細胞株を用いた*in vitro*単クローン抗体作製技術の開発とその有用性についての研究成果を紹介したい．

また，免疫・炎症性疾患における代表的な抗体治療薬としての抗TNF-α抗体について，解説を交えながら多彩な臨床効果を紹介するとともに，その開発の歴史や既存薬剤ではなし遂げられなかった革命的な治療効果について，さらには臨床的立場から今後の生物製剤の展望についても論じる予定である．

最後に，脳梗塞をはじめとする虚血性脳障害に対する抗体医薬の有効性を明らかにする目的で，梗塞病態時の過剰な生体応答に関与する因子としてのヌクレオカインの増悪因子としての意義と特異的単クローン抗体がもたらす劇的な治療効果や作用メカニズムについて紹介し，新たな創薬標的としての可能性について論ずる予定である．

具体的には，本シンポジウムでは，次に掲げた表題を取り上げた（発表順，敬称略）．1）抗体医薬の最前線，オーバービュー（西堀正洋 他，岡山大院医歯薬），2）次世代抗体医薬としてのポテリジェント抗体（設楽研也，協和発酵工業），3）培養B細胞株を用いる*in vitro*抗体作製システムによる有用抗体の創製（金山直樹 他，岡山大院自然科学），4）

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生物学的製剤（抗 TNFα 抗体）による免疫・炎症性疾患治療の現状と展望(杉田尚久，田辺三菱製薬)，5）抗ヌクレオカイン単クローン抗体の脳梗塞治療への応用（森　秀治 他，就実大薬)．

この誌上シンポジウムでは，上記のように抗体医薬研究を巡る多彩なテーマについて最新の研究成果を紹介するが，本シンポジウムを通じて抗体医薬開発の最先端において，将来，われわれが享受し得る医療の選択肢は確実に広がりをみせていることを実感されることであろう．本シンポジウムが抗体医薬分野の研究者のみならず様々な方面に少しでも貢献することができれば，オーガナイザーとして望外の喜びである．今後益々の研究進展のために有益な助言・進言を頂きたいと希望するものである．

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総説

High Mobility Group Box-1を治療標的とする脳梗塞治療

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要約：High Mobility Group Box-1（HMGB1）は，細胞核内に局在するクロマチン結合性の非ヒストンタンパクとして同定され，クロマチン構造の維持機能や転写活性調節，DNA修復等に重要な働きをすると考えられていたが，1999年，Kevin Traceyらの研究グループが敗血症ショックの遅延性メディエータとして再発見し，以後種々の炎症性疾患における関与が次々に示唆されるようになってきた．脳梗塞急性期における血液―脳関門の破綻は，血管内皮細胞，ミクログリア，アストログリア，循環血中白血球の活性化とそれらの相互作用による脳内炎症反応によってもたらされると考えることができるが，このような過程における壊死細胞からのHMGB1の放出は脳内炎症において重要な働きをする可能性がある．最近相次いで報告されてきた脳虚血急性期の脳内HMGB1の動態と，HMGB1を標的としたshRNAや抗HMGB1抗体を用いた治療について，著者らの知見を中心に概説する．

はじめに

脳血管障害は，わが国の死因の第3位を占める疾患であり，そのうち約60%は脳梗塞によるものである．脳梗塞はそれ自身直接の死因となるだけでなく，たとえ一命を取り留めた場合でも重篤な神経後遺症状を残すことが多く，要介護の原因疾患のトップを占めている．従って，患者本人や家族の肉体的・精神的ストレスはもとより，社会経済的な損失と負担も大きなものとなる．したがって，高齢化社会のわが国において，その予防と重症化の防止は喫緊の課題であると言える．1990年代，血栓溶解薬である組織型プラスミノーゲンが臨床治療薬として登場し，大きな期待をもって迎えられた．しかし，有効治療時間帯が短いこと，脳出血のリスクが伴うことが原因し，適用される患者の割合は低い状態が続いている．一方，非常に多くの脳保護薬候補の研究からは，有望な新規薬は未だ見出されていない．

本稿では，これまで指摘された種々の動物モデルと脳梗塞治療薬の評価法に関する問題点を整理し，最近脳梗塞の治療標的分子として注目されるようになってきたHigh Mobility Group Box-1（HMGB1）を中心に最近の進歩について概説する．

1．脳虚血・脳梗塞動物モデルと結果の評価

前臨床研究で有効性が証明された多くの脳梗塞治療薬が，臨床治験においては組織型プラスミノーゲンアクチベータを除き一つとして有効性が見出されなかったという反省に立ち，1999年雑誌Stroke誌に，「前臨床研究においていかに動物モデルを用いた脳梗塞治療薬研究をすすめるべきか」についての勧告記事が掲載された(1)．その中で指摘された項目は，1）適切な治療濃度における用量―反応性の検討，2）有効治療時間帯，3）生理的パラメータの適切なモニタリングと実験のブラインド化，4）独立の2つ以上の研究室での再現実験，5）脳梗塞サイズ以外の神経機能評価法，6）異なった脳梗塞モデルの採用，7）小動物での実験後に大型動物（霊長類）の実験，とまとめることができる．以上の項目は，現在でも変わらぬ重要性を持っており，特に，一過性脳虚血後の脳梗塞モデルと永久閉塞による脳梗塞モデルでは，再灌流障害の寄与，循環血中白血球や血小板の役割，血液凝固系の影響など，梗塞形成機序にも相違する点が存在する可能性がある．したがって，臨床病型の複雑さを考えれば，複数の動物モデルでの実験によって薬物効果を評価すべきであるというのはうなずける．また，ヒト臨床における神経症状を的確に反映できるモデルとして霊長類を用いることに言及されたが，脳梗塞サイズ以外に，神経学的症状を多面的に評価する必要性が強調された．これまでに，前臨床実験あるいは治験で効果が評価されてきた薬物は，表1に示すように非常に多岐にわたって

キーワード：脳梗塞，HMGB1，血液脳関門，サイトカイン，抗体医薬
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