Virchows Arch (1998) 433:375–379 © Springer-Verlag 1998

ORIGINAL ARTICLE

Kuniaki Nakanishi · Fumiko Tajima · Yasuko Nakata
Hiroshi Osada · Katsuhiko Ogata · Toshiaki Kawai
Chikao Torikata · Tatsuko Suga · Kunio Takishima
Takashi Aurues · Tomosumi Ikeda

Tissue factor is associated with the nonbacterial thrombotic endocarditis induced by a hypobaric hypoxic environment in rats

Received: 6 March 1998 / Accepted: 30 April 1998

Abstract High-altitude hypoxia causes a hypercoagulable state. In our previous study on the blood coagulation system in rats, nonbacterial thrombotic endocarditis (NBTE) developed after 4–12 weeks' exposure to the equivalent of 5500 m in altitude. We hypothesized that TF (tissue factor)-producing cells in the cardiac valves might be induced by the hypobaric hypoxic environment (HHE) and then trigger NBTE. A total of 170 male Wistar rats were housed in a chamber at the equivalent of 5500 m altitude for 1–12 weeks. We measured TF activity in the plasma and studied morphological changes in the mitral valves using immunohistochemical and immunoelectrical methods for TF protein and in situ hybridization for TF mRNA. After 4 weeks or more of exposure to HHE, 28 of the 56 surviving rats had developed NBTE. After 4–8 weeks' exposure to HHE, the plasma TF activity level was significantly higher than in control rats. There was a significant correlation between plasma TF activity and the incidence of NBTE. After 1 weeks' exposure to HHE, immunoreactivity for TF protein was detected in foamy macrophages and stromal cells in the cardiac valves. In rats with NBTE, TF protein was present in foamy macrophages and spindle stromal cells and focally present in the extracellular matrix. TF mRNA was detected in some foamy macrophages within the thrombus, TF protein was localized to the rough endoplasmic reticulum and plasma membrane of many macrophages, some fibroblasts, and a few endocardial cells. TF is associated with the pathogenesis of the NBTE induced by exposure to HHE. The accumulation of TF-producing macrophages during exposure to HHE may be responsible for initiating thrombus formation.

Key words Cardiovascular disease · Coagulation · Hypoxia · Thrombosis · Valves

K. Nakanishi (✉) · T. Aurues · T. Ikeda
Division of Environmental Medicine,
National Defense Medical College Research Institute,
Tokorozawa 359, Japan
Tel.: +81-429-95-1626, fax: +81-429-91-1612

F. Tajima · Y. Nakata · H. Osada · K. Ogata
Biochemical Division, Aeromedical Laboratory,
Japan Air Self-Defense Force, Tachikawa 190, Japan

K. Kawai · C. Torikata
Department of Pathology, National Defense Medical College,
Tokorozawa 359, Japan

T. Suga · K. Takishima
Department of Biochemistry, National Defense Medical College,
Tokorozawa 359, Japan

Introduction

High-altitude hypoxia is a well-known cause of both polycythaemia and a hypercoagulable state in humans and animals [4, 7]. A hypobaric hypoxic environment (HHE) produced nonbacterial thrombotic endocarditis (NBTE) in rats after 4–12 weeks' exposure to conditions equivalent to an altitude of 5500 m [7]. Our earlier results suggest that exposure to HHE induces a hypercoagulable state in rats and causes an NBTE that may result in consumption coagulopathy. However, the pathogenesis of the NBTE induced by HHE is still unclear.

Tissue factor (TF) is the major cellular initiator of coagulation protease cascades, and it serves as a receptor and essential cofactor for plasma factors VII/VIIa [1, 8]. Aberrant expression of TF is associated with a variety of clinical diseases [6, 9]. In atherosclerosis, the presence of TF protein in the matrix of the plaque may contribute to the hyperthrombotic state of atherosclerotic vessels [10]. Furthermore, in atherosclerosis, macrophages and monocytes exhibit TF mRNA and protein [10]. However, an association between TF and NBTE has not yet been demonstrated. We hypothesized that TF-producing cells in the cardiac valves may be induced by HHE, and may then trigger NBTE. To test this idea, Wistar rats were housed for up to 12 weeks in a mechanical chamber in an environment that was the equivalent of 5500 m altitude. Using these animals, we set out to evaluate the immunohistochemical and immunoelectrical expression of TF protein, and the in situ hybridization of TF mRNA in lesions of the heart valves.

Methods

We used essentially the same materials and methods as reported previously [7]. Briefly, a total of 170 adult male Wistar rats, approximately 8 weeks old and weighing 250–350 g, were divided into seven groups. Fourteen rats kept in the sea level environment served as controls. Groups of 13–75 rats were each housed in a 5.6×3.0×3.0-m mechanical chamber (Hitachi) and exposed to an HHE equivalent to 5500 m in altitude (380 mm Hg=50.6 kPa) for 1–12 weeks. The oxygen content in the main room was maintained constant at an F_IO_2 of 0.105 to simulate an altitude of 5500 m. The age of the rats when exposure to HHE was initiated was such that all were approximately 20 weeks old at the termination of the experiment. All rats were given commercial chow and tap water ad libitum. The protocols were approved by the Committee on the Ethics of Animal Experiments in the Aeromedical Laboratory, Japan Air Self-Defense Force.

After 1, 2, 4, 6, 8, and 12 weeks of exposure to HHE, blood samples were drawn from the right heart during anaesthesia induced by an intramuscular injection of ketamine hydrochloride (60 mg/kg body weight) before autopsy, and put into plastic tubes containing anticoagulants.

The heart was removed from each rat autopsy and dissected. Any vegetation on the valves was macroscopically examined. Complete necropsy was then performed. For histological study, all the parts of the various organs taken from each rat were fixed separately in 10% formalin. Some cardiac tissues, including the mitral valves from 3 rats, were fixed in periodic lysine-paraformaldehyde (PLP) solution for subsequent immunoelectrical study.

Plasma samples were assayed in duplicate for TF activity using a spectrophotometric method with the chromogenic synthetic substrate (S-2765) [3]. A mixture of plasma from the normal human pool of plasma and TF derived from human placental tissue thromboplastin (HPT: Behringwerke) was used as a TF-positive standard control.

For immunohistochemistry, the indirect immunoperoxidase method was applied to deparaffinized sections. A primary rabbit polyclonal antibody against TF (American Diagnostica; diluted 1:20) and a horseradish peroxidase-labelled secondary antibody to rabbit immunoglobulins (Chemicon International; diluted 1:250) were used for this purpose.

For in situ hybridization, deparaffinized sections were treated with 0.2 N HCl for 20 min, then incubated in 2×SSC for 10 min at 37° C, and finally incubated in 10 μg/ml proteinase K for 10 min at 37° C. Sections were subsequently postfixed in 4% paraformaldehyde for 5 min, then incubated for 10 min in 0.1 mol/l triethanolamine buffer, pH 8.0, containing 0.25% (vol/vol) acetic anhydride to prevent nonspecific binding due to oxidation of the tissue. The rat TF cDNA probe (prat TF 2375) was a 772-bp fragment subcloned into the EcoR1 site of pGEM-9Z (Promega; a kind gift from Dr. N. Mackman, Departments of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, Calif.). An antisense probe and the corresponding sense probe were labelled with digoxigenin using SP6 and T7 polymerase, respectively, by means of an RNA labelling kit (Boehringer Mannheim). Hybridization was carried out overnight at 42° C in 50% (vol/vol) deionized formamide, 5× Denhardt's solution, 5% (wt/vol) dextran sulfate, 2× SSC, 0.3 mg/ml salmon sperm DNA, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.01 μg/ml digoxigenin-labelled probes. After a final stringency wash had been carried out at 37° C in 2× SSC for 10 min, hybridization was detected immunohistochemically. Sections were then incubated with a monoclonal antibody to digoxigenin (Boehringer Mannheim) followed by rabbit anti-mouse immunogloblin, an APAAP complex (Dakopatts), and a nitroblue tetrazolium substrate solution.

For immunoelectron microscopy, immunostaining for TF protein was again performed by the indirect antibody method. After immersion in 10% nonimmunized goat serum, frozen sections 6 mm thick were incubated first with anti-TF antibody for 24 h at 4° C, and then overnight with the horseradish-peroxidase labelled Fab fragment of the secondary antibody (Amersham; diluted 1:50). After fixation in 1% glutaraldehyde, the specimens were reacted with 0.03% 3-3′-diaminobenzidine tetrahydrochloride, postfixed in 1% osmium tetroxide for 1 h, and embedded in Epon. Ultrathin sections were observed under the electron microscope without being stained.

The results are expressed as the mean±standard error of the mean (SEM). Scheffé's test was applied to the data when significant *F*-ratios were obtained in an analysis of variance (ANOVA). Furthermore, the correlation between the incidence of NBTE and plasma TF activity was assessed using Pearson's correlation coefficient test with the incidence of NBTE considered as continuous data.

Results

After 4 weeks or more of exposure to HHE, 28 of the 56 surviving rats studied here had developed NBTE. The incidence of NBTE at 4, 6, 8, and 12 weeks of exposure to HHE was 33% (5/15 rats), 29% (5/17), 65% (11/17), and 100% (7/7), respectively.

After 4–8 weeks' exposure to HHE TF activity in the plasma (maximal level: 1419±287 μg/ml HPT at 4 weeks) was significantly higher than the level detected in control rats (65±24 μg/ml HPT; $P<0.05$; Fig. 1). There was a significant correlation between the plasma TF activity and the incidence of NBTE ($P=0.009$).

Examination of the immunoreactivity for TF protein in control rats revealed weak staining of myocardial cells, but such staining was not detected in the endocardial and stromal cells of the valves. After 1 week's exposure to HHE, significant immunoreactivity for TF protein was already detectable in a few foamy macrophages and stromal cells, and a small proportion of the extracellular matrix within the valves (Fig. 2a). In rats with NBTE following 4–12 weeks of HHE, TF protein was positive in many foamy macrophages, some spindle stromal cells and a few endocardial cells, and focally positive in the extracellular matrix in the thrombus (Fig. 2b).

In rats with NBTE after 4 weeks' exposure to HHE, TF mRNA was detected in foamy macrophages in the thrombus (Fig. 2c), but it was not detected in the cardiac valves of either control rats or HHE rats after 1–2 weeks' exposure to HHE.

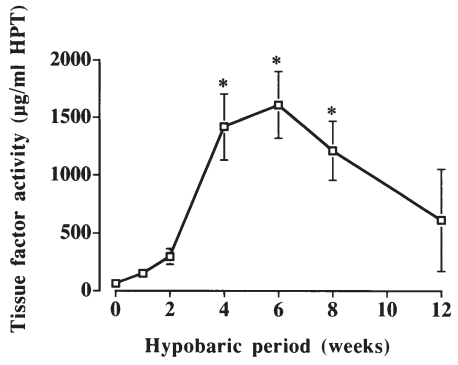

Fig. 1 Activity of tissue factor (TF) using a spectrophotometric method. TF derived from human placental tissue thromboplastin (*HPT*) was used as a TF-positive standard control. (*, $p<0.05$).

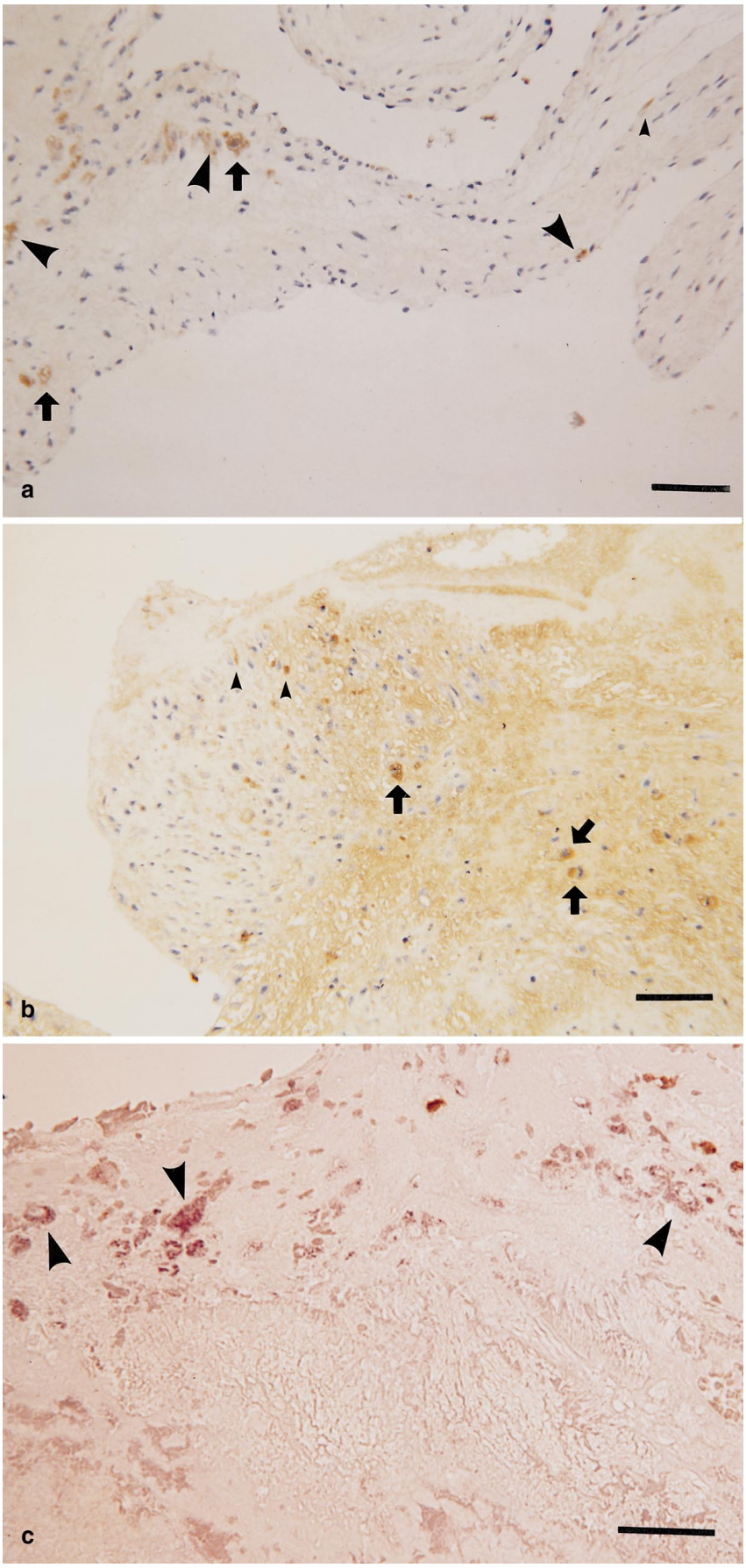

Fig. 2a–c Expression of TF protein and TF mRNA in mitral valve after exposure to a hypobaric hypoxic environment (HHE). **a** Mitral valve after 1 week's exposure to HHE. Staining is present in a few macrophages (*arrows*) and stromal cells (*small arrowhead*) and a small amount of the matrix (*large arrowheads*). *Bar* 100 µm. **b** Mitral valve thrombus after 4 weeks' exposure to HHE, showing TF protein in macrophages (*arrows*) and stromal cells (*small arrowheads*), and focally in the extracellular matrix. *Bar* 100 µm. **c** A section hybridized with an antisense probe for rat TF mRNA: foamy macrophages (*arrowheads*) in the thrombus formed after 4 weeks' exposure to HHE show the signal for TF mRNA. *Bar* 50 µm

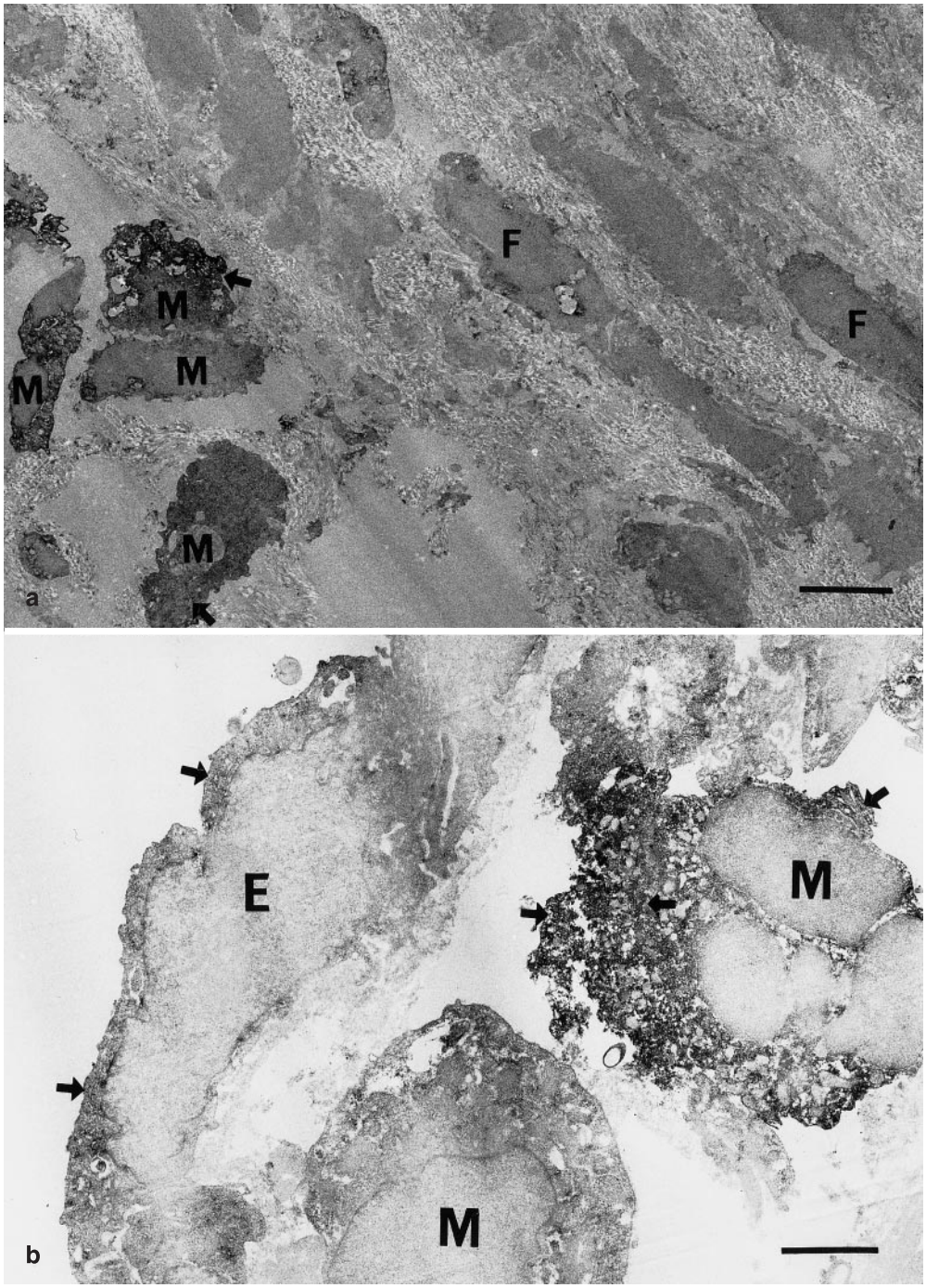

Fig. 3a, b Immunoelectron micrograph of thrombotic lesion of mitral valve stained for TF. Tissue was taken from rats after 4 weeks' exposure to HHE. **a** TF protein is distributed throughout the cytoplasm, particularly on the rough endoplasmic reticulum (*arrows*), and on the plasma membrane of foamy macrophages (*M*) and in the cytoplasm of some fibroblasts (*F*). *Bar* 6 μm. **b** TF protein can be seen in the cytoplasm, particularly on the rough endoplasmic reticulum (*arrows*), and on the plasma membrane of macrophages (*M*) and of one endocardial cell (*E*). *Bar* 2 μm

Immunoelectron micrography revealed that TF protein was distributed throughout the cytoplasm, particularly on the rough endoplasmic reticulum, of foamy macrophages, and also on their plasma membrane (Fig. 3). Some fibroblasts and a few endocardial cells also showed TF protein in patches in the cytoplasm.

Discussion

Pathological activation of coagulation is the key feature in thrombotic disease. As a potent initiator of caogulation, TF is believed to have a critical role in haemostasis and thrombogenesis. However, no previous study has investigated the relationship between TF and the pathogenesis of NBTE, particularly in the early stages of NBTE. In our previous experiment on rats exposed to HHE for up to 12 weeks, we demonstrated NBTE after 4 weeks' exposure. In the present study, TF activity in the plasma

increased greatly after exposure to HHE, and it showed a significant correlation with the incidence of NBTE. TF protein was found in foamy macrophages and stromal cells of cardiac valves in the early stages of NBTE. Moreover, TF mRNA was detected in foamy macrophages in rats with NBTE. In the light of these results, we suggest that TF is associated with the pathogenesis of the NBTE induced by exposure to HHE, and that the macrophages and stromal cells that produce TF after exposure to HHE may be candidates for the role of the cell(s) responsible for initiating thrombogenesis in the this type of NBTE.

The results of our study also support previous suggestions that TF has a major functional role in contact activation pathways in haemostasis and thrombogenesis [1,8]. TF does not appear to be present in the cardiac valves of normal rats or in the putative site of contact activation under normal conditions [10]. When the detachment of endocardial cells from the mitral valve was found after 1 week's exposure to HHE, TF protein was already induced in macrophages and stromal cells under the endocardial cells. In fact, although monocytes and macrophages exhibit little constitutive expression of TF under normal conditions, they can be induced to express TF in vitro by a variety of stimuli [6, 9]. Furthermore, one recent study demonstrated thrombosis in murine pulmonary vessels after 8 h exposure to normobaric hypoxia (oxygen tension, 6%), and the authors suggested that TF expression may trigger the local deposition of fibrin [5].

TF-initiated thrombosis is reported to be unlikely to result from simple injury to the normal endothelium leading to a loss of cellular integrity, unless there has been previous activation [2]. Therefore, it can be presumed that certain mediators are implicated in the thrombogenic process that is partly responsible for the NBTE induced by HHE. Indeed, endogenous inflammatory mediators such as interleukin-1, tumour necrosis factor (TNF) and endotoxin and immune complexes, have been reported to induce endothelial cells to express TF [1, 6]. In our study, a few endocardial cells were expressing TF protein in the thrombus after 4 weeks' exposure to HHE (as revealed by immunohistochemistry and immunoelectron microscopy). In the early stages of exposure to HHE, however, TF protein was produced mainly be macrophages and stromal cells within the valves.

We found that expression of TF protein was already detectable by immunohistochemistry after 1 week's exposure to HHE, whereas TF mRNA was not detectabed by in situ hybridization at this time. The reason for this discrepancy is unclear. One possibility is that the sensitivities of the anti-TF protein antibody and the TF mRNA probe may be different. Moreover, our in situ hybridization procedure may be not sufficiently sensitive to detect the expression of TF mRNA because of our use of formalin-fixed paraffin-embedded tissues. In addition, we have to consider the possibility that the levels of TF mRNA in these cells may be too low to be detected by digoxigenin-labelled probes.

Finally, TF production by macrophages and stromal cells was localized to within the mitral valve at an early stage of NBTE, suggesting that the pathogenesis of NBTE may be triggered by TF expression in the parenchyma of the valve. Furthermore, we found that the TF activity in the plasma increased more than 20-fold in the first 4 eeks of HHE. This increase in plasma TF activity may be the result of increased functional activity of TF in one or more organs. In fact, TF is widely expressed in the extravascular cells of many tissues, and it is particularly abundant in brain and lung [2]. For this reason, more research is needed to reveal the effects of HHE on TF in the various organs of the body.

Acknowledgements This research was supported in part by a grant from the Ichiro Kanehara Foundation, Tokyo, Japan. We are indebted to Dr. Nigel Mackman for providing the rat TF probe, and to Dr. Robert Timms for correcting the English version of the manuscript.

References

1. Davie EW, Fujikawa K, Kisiel W (1991) The coagulation cascade: initiation, maintenance, and regulation. Biochemistry 30:10363–10370
2. Drake TA, Morrissey JH, Edgington TS (1989) Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis. Am J Pathol 134:1087–1097
3. Fukuda C, Nakagawa M, Iijima K, Nakamura K (1993) Qualitative analysis of activities of a tissue factor-like substance in the plasma. Med Sci Res 21:427–429
4. Heath D, Williams DR (1989) High-altitude medicine and pathology. London, Butterworths
5. Lawson CCA, Yaan SD, Yan SF, Liao H, Zhou YS, Sobel J, Kisiel W, Stern DM (1997) Monocytes and tissue factor promote thrombosis in a murine model of oxygen deprivation. J Clin Invest 99:1729–1738
6. Mackman N (1995) Regulation of the tissue factor gene. FASEB J 9:883–889
7. Nakanishi K, Tajima F, Nakata Y, Osada H, Sugiyama K, Maruta H, Kawai T, Suzuki M, Torikata C (1997) Hypercoagulable state in a hypobaric, hypoxic environment causes nonbacterial thrombotic endocarditis in rats. J Pathol (Lond) 181:338–346
8. Nemerson Y (1988) Tissue factor and hemostasis. Blood 71:1–8
9. Ruw W, Edgington TS (1994) Structural biology of tissue factor, the initiator of thrombogenesis in vivo. FASEB J 8:385–390
10. Wilcox JN, Smith KM, Schwartz SM, Gordon D (1989) Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci USA 86:2839–2843