Volume 298, number 1, 21–24 FEBS 10702 February 1992

Production of endothelin-1 and big-endothelin-1 by pleural mesothelial cells

Masayoshi Kuwahara[a], Maki Kuwahara[b] and Nobuhiro Suzuki[c]

[a]*Department of Comparative Pathophysiology, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan,* [b]*Division of Toxicology, Institute of Environmental Toxicology, Suzuki-cho 2-772, Kodaira-shi, Tokyo 187 Japan* and [c]*Tsukuba Research Laboratories, Takeda Chemical Industries Ltd., 7 Wadai, Tsukuba Ibaraki 300-42, Japan*

Received 4 December 1991

Immunoreactive endothelin-1 (ET-1) and big-endothelin-1 (big-ET-1) were detected in conditioned medium of cultured rat pleural mesothelial cells by using sensitive sandwich-type enzyme immunoassays. The amount of both ET-1 and big-ET-1 increased time-dependently. In both instances, maximal amount was detectable in conditioned medium obtained after 72 h in culture (ET-1: 117.1 ± 30.1 pg/10^6 cells; big-ET-1: 2.4 ± 2.1 pg/10^6 cells). Fetal calf serum markedly stimulated the production of both ET-1 and big-ET-1.

Endothelin; Big-endothelin; Mesothelial cell; Pleura; Rat

1. INTRODUCTION

Endothelin-1 (ET-1) was originally isolated from the conditioned medium of cultured porcine aortic endothelial cells as a potent vasoconstrictor peptide with 21 amino acid residues [1]. Cloning and sequencing of prepro-ET-1 cDNA have revealed the identity of human and porcine ET-1s [1,2]. These studies have also suggested the involvement of a precursor peptide, 'big-endothelin-1 (big-ET-1)', in the biosynthetic pathway of ET-1. The conversion from big-ET-1 to ET-1 was essential for full physiological activity. Recent studies have documented that besides aortic endothelium, other types of cells such as airway epithelial cells [3], mesangial cells [4], spinal cord [5], and epithelial-like tumer cells [6] may also generate ET.

Mesothelial cells which also have epithelial-like characters are a biological barrier between an organ and the external 'envelope' and may have functions of transport, equilibrium maintenance, and protection. From a biological point of vieuw, therefore, it could be interesting to know more about the metabolism and physiology of these cells. Although there is no observation concerning ET production on mesothelial cells, it is conceivable that mesothelial cells may also generate ET. The present study was designed to test this possibility. For this purpose, mesothelial cells were maintained in vitro and their conditioned medium was evaluated using a sensitive sandwich-type enzyme immunoassay (sandwich-EIA) for ET-1 and big-ET-1 [6,7]. Our findings indicated that cultured mesothelial cells produced both immunoreactive ET-1 and big-ET-1.

Correspondence address: M. Kuwahara, Department of Comparative Pathophysiology, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan. Fax: (81) (3) 5800-6925.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Rat pleural mesothelial cells were routinely maintained in complete culture medium, which consists of Dulbecco's minimal essential medium (DMEM; Gibco) with 10% fetal calf serum (FCS; Gibco), 10^5 U/l penicillin, and 100 mg/l streptomycin, at 37°C in humidified environment containing 5% CO_2 as previously described [8]. Eight to 10 passage number of cells were used in this experiment. When confluence was reached (55 cm^2), the cells were washed three times with Hanks' balanced salt solution (HBSS) and incubated with either 10 ml of serum-free medium or medium containing 10% FCS for up to 72 h. Conditioned medium was collected at defined culture time, and frozen in aliquots at −70°C. After collecting conditioned medium, viable cell numbers were confirmed by Trypan blue dye exclusion.

2.2. *Sandwich-EIA*

The amount of immunoreactive ET-1 and big-ET-1 in the conditioned medium was determined by sandwich-EIA methods as described previously [6,7]. Briefly, AwETN40-coated microtest plates were incubated at 4°C for 24 h with 100 μl/well of standard ET-1, human big-ET-1, or the sample to be tested in buffer E (0.02 M phosphate buffer, pH 7.0, containing 10% Block Ace, 2 mg/ml of bovine serum albumin (BSA), 0.4 M NaCl, and 2 mM EDTA). After being washed with PBS, the plates were subjected to the reaction with 100 μm/well of HRP-labeled anti-ET-1 (15–21)Fab' or HRP-labeled anti-human big-ET-1 (22–38)Fab' in buffer C (0.02 M phosphate buffer, pH 7.0, containing 10 mg/ml of BSA, 0.4 M NaCl, and 2 mM EDTA) for 24 h at 4°C. The plates were washed with PBS, and the bound enzyme activity was measured using a TMB microwell peroxidase system (KPL Inc., Gaithersburg, USA).

2.3. *Reverse-phase HPLC*

Conditioned medium cultured with 10% FCS for 72 h (20 ml) was acidified with acetic acid and applied to a Sep-pak C_{18} cartridge

(Waters Associates, Milford, MA) as previously described [7]. The adsorbed material was eluted with 5 ml of acetic acid/ethanol/H_2O (4:86:10) and concentrated. The concentrated sample was separated on a TSK ODS-80 (4.6 × 250 mm; Tosoh, Co. Ltd., Japan) column. The solvents used were: (A) 5% CH_3CN containing 0.05% TFA; and (B) 60% CH_3CN containing 0.05% TFA. In the elution, the B concentration was lineary elevated 0–40% over 5 min, 40–65% over 20 min, and then 65–100% over 5 min at a flow rate of 1.0 ml/mim [6]. Each fraction (0.5 ml) was lyophilized, reconstituted with the EIA buffer, and subjected to the EIAs for ET-1 and big-ET-1.

3. RESULTS AND DISCUSSION

We have detected immunoreactive ET-1 and big-ET-1 in conditioned medium from cultured rat pleural mesothelial cells whose viability, based on Trypan blue dye exclusion, always exceeded 95% (Fig. 1). These mesothelial cells produced more immunoreactive ET-1 than big-ET-1. The production of immunoreactive ET-1 was progressively increased in culture time-dependent manner. The amount of immunoreactive big-ET-1 was slightly increased with culture time. In both instances, maximal amount was detectable in conditioned medium obtained after 72 h in culture (immunoreactive ET-1: 117.1 ± 30.1 pg/10^6 cells; immunoreactive big-ET-1: 2.4 ± 2.1 pg/10^6 cells). In addition, we examined the effects of FCS on the production of immunoreactive ET-1 and big-ET-1 in the conditioned medium. As shown in Fig. 2, FCS markedly stimulated the production of both immunoreactive ET-1 and big-ET-1. Because levels of immunoreactive ET-1 and big-ET-1 in control medium both serum-free and containing FCS were below the limits of detection, immunoreactive ET-1 and big-ET-1 detected in conditioned medium were not components of FCS. Immunoreactive ET-1 and big-ET-1 were also characterized by reverse-phase HPLC (Fig. 3). Immunoreactive ET-1 and big-ET-1 appeared at the elution positions corresponding to synthetic ET-1 and human big-ET-1, respectively. These findings indicated that cultured mesothelial cells generated both immunoreactive ET-1 and big-ET-1.

To our knowledge, mesothelial cells have not been shown previously to elaborate both ET and big-ET. Giaid and associates [9] recently have shown that pulmonary endocrine cells of developing human lung mainly expressed ET-like immunoreactivity. Radioimmunoassay has revealed the presence of high concentration of ET immunoreactivity in the porcine and rat lung [10,11]. Moreover, ET receptor binding sites have been detected in the cardiovascular and respiratory systems of mammals [12–14]. In these studies, however, there was no investigation for ET about mesothelial cell layer in respiratory tract.

It seems that mesothelial cells may also respond to

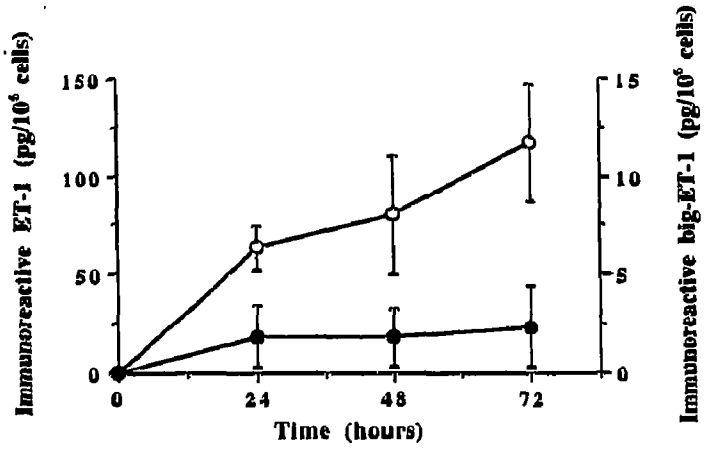

Fig. 1. Time course illustrating the progressive production of immunoreactive ET-1 and big-ET-1 in conditioned medium obtained from rat pleural mesothelial cell cultures. Open circles = immunoreactive ET-1; solid circles = immunoreactive big-ET-1. Mean ± SE of 3 experiments for each category.

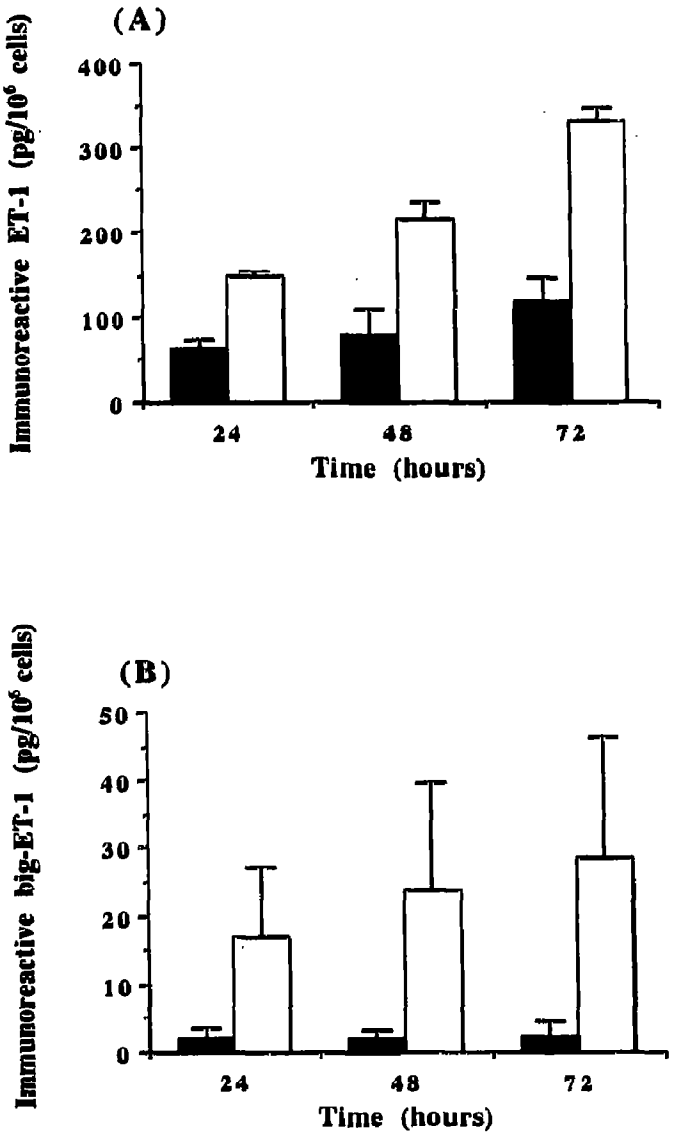

Fig. 2. Effects of FCS on the production of immunoreactive ET-1 (A) and big-ET-1 (B) in conditioned medium obtained from rat pleural mesothelial cell cultures. Mean ± SE of 3 experiments for each category. Solid bars = pleural mesothelial cells cultured without FCS; open bars = pleural mesothelial cells cultured with FCS.

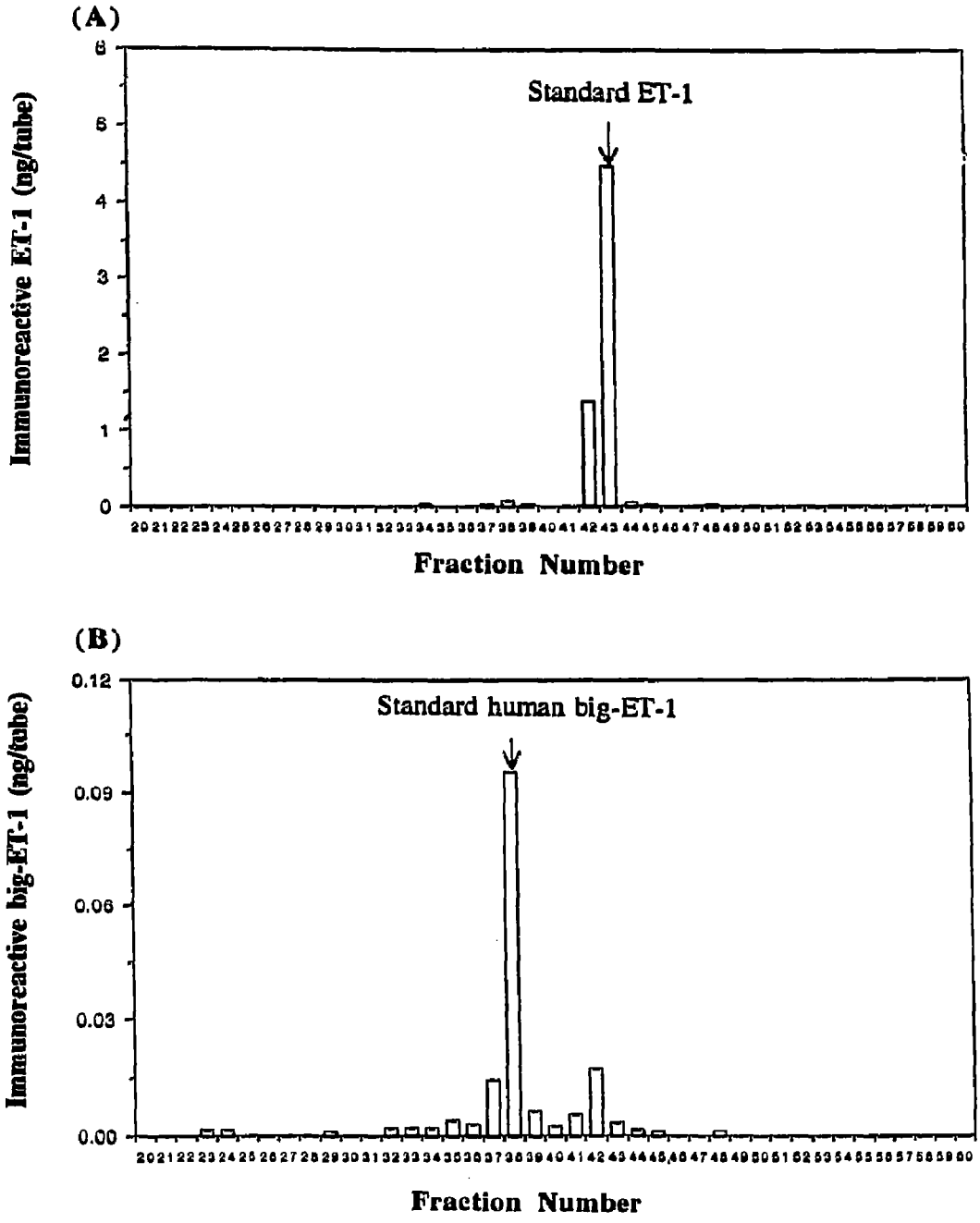

Fig. 3. Reverse-phase HPLC of the culture conditioned medium of rat pleural mesothelial cells on a TSK ODS-80TM column. Aliquots of the fractions were subjected to sandwich-EIAs for ET-1 (A) and big-ET-1 (B). Arrows indicate the elution positions of synthetic ET-1 and human big-ET-1.

serum factors such as transforming growth factor-β and thrombin which induce preproET-1 gene expression in endothelial, epithelial, and mesangial cells [4,15–18], because production of ET in mesothelial cells was enhanced by the addition of FCS. Moreover, ET acts as a potent mitogen in fibroblasts [19–21]. The growing view, therefore, is that the ability of mesothelial cells to produce ET may play a role in the reparative response of injury to provide a signal for fibroblast proliferation in submesothelial layer. Further ET may have the function of regulating the transport and equilibrium maintenance in the serous surfaces.

In summary, we have shown that cultured rat pleural mesothelial cells can generate immunoreactive ET-1 and big-ET-1. Although further study will be necessary to determine the physiological and pathophysiological role of ET peptides in the serous surfaces, mesothelial cells may play an important role in these regions.

Acknowledgements: This work was supported in part by Grant-in-Aid

(no. 03760215) from the Scientific Research Fund on the Ministry of Education, Science and Culture, Japan.

REFERENCES

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[2] Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M. and Masaki, T. (1988) FEBS Lett. 231, 440–444.
[3] Black, P.N., Ghatei, M.A., Takahashi, K., Bretherton-Watt, D., Krausz, T., Dollery, C.T. and Bloom, S.R. (1989) FEBS Lett. 255, 129–132.
[4] Zoja, C., Orisio, S., Perico, N., Benigni, A., Morigi, M., Benatti, L., Rambaldi, A. and Remuzzi, G. (1991) Lab. Invest. 64, 16–20.
[5] Shinmi, O., Kimura, S., Yoshizawa, T., Sawamura, T., Uchiyama, Y., Sugita, Y., Kanazawa, I., Yanagisawa, M., Goto, K. and Masaki, T. (1989) Biochem. Biophys. Res. Commun. 162, 340–346.
[6] Suzuki, N., Matsumoto, H., Kitada, C., Kimura, S. and Fujino, M. (1989) J. Biochem. 106, 736–741.
[7] Suzuki, N., Matsumoto, H., Kitada, C., Masaki, T. and Fujino, M. (1989) J. Immunol. Methods 118, 245–250.
[8] Kuwahara, M., Kuwahara, M., Bijwaard, K.E., Gersten, D.M., Diglio, C.A. and Kagan, E. (1991) Am. J. Respir. Cell Mol. Biol. 5, 256–264.
[9] Giaid, A., Polak, J.M., Gaitonde, V., Hamid, Q.A., Moscoso, G., Legon, S., Uwanogho, D., Roncalli, M., Shinmi, O., Sawamura, T., Kimura, S., Yanagisawa, M., Masaki, T. and Springall, D.R. (1991) Am. J. Respir. Cell Mol. Biol. 4, 50–58.
[10] Kitamura, K., Tanaka, T., Kato, J., Eto, T. and Tanaka, K. (1989) Biochem. Biophys. Res. Commun. 161, 348–352.
[11] Matsumoto, H., Suzuki, N., Onda, H. and Fujino, M. (1989) Biochem. Biophys. Res. Commun. 164, 74–80.
[12] Kanse, S.M., Ghatei, M.A. and Bloom, S.R. (1989) Eur. J. Biochem. 182, 175–179.
[13] Koseki, C., Imai, M., Hirata, Y., Yanagisawa, M. and Masaki, T. (1989) Am. J. Physiol. 256, R858–R866.
[14] Power, R.F., Wharton, J., Zhao, Y., Bloom, S.R. and Polak, J.M. (1989) J. Cardiovasc. Pharmacol. 13, S50–S56.
[15] Kurihara, H., Yoshizumi, M., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T., Hamaoki, M., Kato, H. and Yazaki, Y. (1989) Biochem. Biophys. Res. Commun. 159, 1435–1440.
[16] Moon, D.G., Morgan, M.J., Andersen, T.T., Krystek, S.R., Fenton, J.W. and Malik, A.B. (1989) Proc. Natl. Acad. Sci. USA 86, 9529–9533.
[17] Ohta, K., Hirata, Y., Imai, T., Kanno, K., Emori, T., Shichiri, M. and Marumo, F. (1990) Biochem. Biophys. Res. Commun. 169, 578–584.
[18] Schini, V.B., Hendrickson, H., Heublein, D.M., Burnett, J.C. and Vanhoutte, P.M. (1989) Eur. J. Pharmacol. 165, 333–334.
[19] Brown, K.D. and Littlewood, C.J. (1989) Biochem. J. 263, 977–980.
[20] Muldoon, L.L., Pribnow, D., Rodland, K.D. and Magun, B.E. (1990) Cell Regulation 1, 379–390.
[21] Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K. and Masaki, T. (1989) J. Biol. Chem. 264, 7856–7861.