INTERACTION OF ENDOTHELIAL CELLS AND MYOCYTES IN CARDIOVASCULAR SYSTEM

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Abstract:

We isolated vascular endothelial cells and smooth muscle cells from human donors of various ages and investigated their ability to secrete ET-1 and that to contract induced by ET-1. Expression of preproET-1 mRNA was enhanced with age of donor and secretion of ET-1 was also increased. Smooth muscle cells in culture contracted with changes of intracellular Ca\textsuperscript{2+} concentration as a response to ET-1 stimulation. Senescent cells less-responded to ET-1 stimulation than young cells. Senescent cells were suggested to have receptors for ET-1 fewer than young. Smooth muscle cells in aorta of the elderly were stained immunohistologically with anti-ET-1 antibody. When analyzed the culture medium of smooth muscle cells, it was found that smooth muscle cells synthesize and secrete ET-1. This suggests the possibility of autocrine action of ET-1 on smooth muscle cells. On the other hand, ET-1 receptor exists on the surface of endothelial cell itself, suggesting the possibility of cross-talk between endothelial cells and smooth muscle cells. Similar possibility can exist in heart: ET-1 stimulates the rhythmic heart-beat of cardiac myocytes and is produced by the cardiac myocyte itself under the condition of hypoxia.

Key words: endothelin, endothelial cell, smooth muscle cell, cardiac myocyte, aging

Endothelin (ET) \textsuperscript{1} is a 21-amino acid peptide, which has originally been identified in endothelial cell culture medium as a novel and potent vasoconstrictor\textsuperscript{1}. There is an ET gene family comprising three types of ET: ET-1, ET-2 (VIC in rodent), ET-3. ET-1 has been detected in many organs, such as the lung, kidney and brain, and has various biological functions including vasoconstriction\textsuperscript{1-4}. The function of ET-2 has not been fully elucidated except for vasoconstriction. The immunoreactive ET-3 has been found in the brain and intestine of rats\textsuperscript{5}, and ET-3 expression has been detected in the placenta\textsuperscript{6} and in the cultured breast epithelial cells\textsuperscript{7}. Recent gene-knocked out experiments showed that deficiency of ET-1 gene causes the abnormal formation of branchial arch\textsuperscript{8} and ET-3 is essential in the development of neural crest-derived cell lineage, i.e., epidermal melanocytes and enteric neurons\textsuperscript{9}. Vasoactive intestinal contractor (VIC), a novel member of the ET family in rodent, is a potent intestinal contractor compared with ET-1\textsuperscript{10}, and cDNA of a precursor for VIC was cloned from mouse intestine\textsuperscript{11}.

The endothelial cell were paid attention to its abilities of anti-thrombosis and to regulate permeability of substances. After the discovery of ET-1, however, its ability to secrete hormones has been taken notice of. ET-1 produced by
vascular endothelial cells acts on smooth muscle cells in vasculature, induces contraction of them and elevates blood pressure. There are two possibilities that ET-1 is secreted into lumen, transported by the blood stream and acts on the remote vasculature as an endocrine factor and that ET-1 passes through the basal membrane and acts on smooth muscle cells existing near the endothelial cells secreting it as a paracrine factor.

ET also displays a powerful cardiotonic property. It has been reported that cardiac membrane and cardiac myocytes have high density receptors for ET-1 on their cell membranes, and that ET-1 possessed inotropic effect on myocardium in vivo. Therefore, hearts have been regarded as one of the target organs of ET-1. An elevation of ET-1 level in the circulation has been reported in patients with myocardial infarction or cardiogenic shock. These reports suggest that ET-1 may relate to some heart failures. Messenger RNA of ET-1 was detected in the heart tissue, however, it still remains unclear what type of cell contributed the expression of mRNA of ET-1 in a heart tissue and even ET-1 has not been detected in intact heart tissues in vivo.

It has been reported that ET can stimulate the incorporation of [3H] thymidine into vascular smooth muscle and endothelial cells, fibroblasts, melanocytes and other type of cells. On the contrary, there are some reports of unstimulating effect on DNA synthesis and/or cell growth by ET on vascular smooth muscle cells and other type of cells and even the report of suppressive activity on thymidine incorporation by ET-1 in a heart tissue and even ET-1 has not been detected in intact heart tissues in vivo.

Vascular endothelial cells have been found to exhibit a finite life span. Culture of endothelial cells from elderly persons contains larger cells, which is a characteristic of cells aged in vitro, and such large cells indeed become the majority in monolayers of endothelial cells lining the vascular wall of elderly people. Furthermore, a negative correlation between in vitro life span and donor age has been observed. Hypertension is often observed in the elderly. As we describe below, ET-1 is produced more in senescent endothelial cells. Thus, hypertension of the elderly might relate to the higher production of ET-1 in senescent cells.

Aging dependence of endothelin-1 expression

We investigated in vivo distribution of ET-1 by immunostaining the sections of aorta from various ages of donor. Aortic endothelial and medial smooth muscle cells of the human fetus were not immunoreactive to anti-ET-1 antibody, but those from a full-term infant were positive for ET-1 with a gradual increase in the older subjects up to 20-year-old. The intensity of immunoreactive ET-1 was always much greater in the endothelial cells than that in the smooth muscle cells. The immunostaining intensity of endothelial cells on aortic tissue sections from elderly subjects was equal to that from the younger subjects. This is a sharp contrast to prostacyclin synthesis in that the aortic endothelial cells of the young group synthesized the largest amount of prostacyclin in vitro and were most intensely immunostained, with progressive decrease in the older groups. The intimal and medial smooth muscle cells were steadily but less intensely immunostained in their cytoplasms than endothelial cells by anti-ET-1 antibody. In situ hybridization of the aortic sections was carried out to identify the site of ET-1 synthesis. The signals showing the existence of ET-1 mRNA were most intense on the endothelial cells from the elderly subject. Fetal aortic endothelial cells revealed the least signals, indicating a developing but still immature gene transcription system. Interestingly, positive signals, though milder than those of endothelial cells, were noted in most of the intimal and medial smooth muscle cells.

To clarify the regulation level of increase of ET expression, we determined the ET-1 mRNA levels of cultured aortic endothelial cells from 5-, 50- and 76-year-old donors by in situ hybridization. The levels of 5- and 50-year-old cells are not different significantly from each other, but in case of 76-year-old cells it is about 2 times higher than the rest. Thus, the expres-
sion of ET-1 gene is up-regulated during cellular aging in vivo and it is suggested that the synthesis of ET-1 peptide is regulated, at least in part, at the level of mRNA. Furthermore, Northern blot analysis of human umbilical vein endothelial cells showed slight increase of expression of ET-1 mRNA during in vitro aging\(^{35}\). The increase in expression of ET-1 mRNA during in vitro cellular aging was also shown by in situ hybridization. The pattern of increase in mean grains per cell with increase in population doubling level (PDL) was very similar to that obtained from Northern analysis. These data suggest that the increased expression of ET-1 during aging in vivo at both the levels of mRNA and peptide is caused by aging of endothelial cells and this confirms that the nature of the cellular aging is the using up of division potential as described in the section of FN.

Cultures of aortic endothelial cells from infancy to an elderly subject were used to measure the amount of ET-1 peptide released into the media by a radio-immunoassay method\(^{33}\). ET-1 synthesis was invariably low in subjects under the age of 50, but endothelial cells from the elderly subjects synthesized much more ET-1. The highest value was obtained from the oldest subject, however, ET-1 synthesis by umbilical vein endothelial cells was lowest. When the 11 subjects were divided into two groups at the age of 50, ET-1 synthesis by the older group over the age 50 was significantly higher than that of the younger group. The increase in level of ET-1 peptide synthesis during in vitro aging is also observed when cells have reached over 48 PDL. The increasing pattern is very similar to that obtained from the quantification of ET-1 peptide for aortic endothelial cells aged in vivo. The pattern is also similar to those obtained from in situ and Northern hybridization analyses. Thus, the increase in synthesis of ET-1 peptide is mainly regulated at the level of mRNA.

Smooth muscle cells were shown to be positive to ET-1 synthesis by immunostaining and in situ hybridization in tissues as described above. To further confirm that smooth muscle cells are really synthesizing ET-1, the culture media of aortic smooth muscle cells from neonate and 71-year-old subjects were checked by radio-immunoassay\(^{32}\). The result clearly showed that smooth muscle cells synthesize ET-1, though their synthetic rate is much less than that of endothelial cells and almost same in young and old subjects.

The relationship between increased ET-1 synthesis and aging was demonstrated in some, but not all, of the elderly subjects. Interestingly, the ET-1 synthesis rate significantly increased in subjects over age 50. This result indicates that ET-1 is a possible factor for hypertension that is often seen in the elderly population. However, the circulating ET-1 peptide levels are said to be low\(^{36-38}\), though a report has revealed a close relationship between the increased plasma level of ET-1 and coronary spasm\(^{39}\). ET-1 enhances the response to noradrenaline and 5-hydroxytryptamine in isolated arteries of the rat and man\(^{38,40,41}\). This indirect action of ET-1 may be more important\(^{42,43}\). Furthermore, the sensitivity of vascular smooth muscle cells to ET-1 decreases with age\(^{40}\). Thus, the higher production rate of ET-1 by aged people may reflect a feedback mechanism of the cardiovascular system to compensate for the lowered sensitivity. The relationship between blood concentration of ET peptide and blood pressure is not clear. However, the higher expression of ET-1 mRNA in senescent cells makes cells produce more ET-1 peptide than young cells and the higher production of ET-1 peptide may relate to the higher level of ET-1 concentration in blood observed in the elderly, though the relationship between ET-1 synthesis and hypertension was not statistically evaluated because of shortage in the number of cases. Since ET-1 is a strong vasoconstrictor, it might cause the high pressure in the elderly by constricting capillary and other vessels. Again, the precise relationship between ET-1 synthesis and hypertension remains unclear.

Growth dependence of endothelin-1 synthesis

One of the characteristics of cellular aging is the decreases in labeling index and percentage
of S-phase cell in cell cycle. Most of the cell specific functions are expressed in non-S phase of confluent culture and most of the markers of cellular aging are closely related to growth arrest. Particularly, S-phase-specific gene expression decreases in senescent culture as in quiescent young cell culture. We consider that an aging marker, which is not the second effect of growth retardation, is a valuable mean to examine the mechanism of aging. Therefore, we examined whether the enhanced synthesis of ET-1 mRNA and peptide are merely the reflection of increase in non-S-phase cells with \textit{in vitro} aging\textsuperscript{35}. Young endothelial cells were seeded at low density, grew logarithmically and reached saturation density until day 6. ET-1 peptide concentration of conditioned medium of the culture was measured at alternative day. The results suggested that the sparsely growing cells secrete more ET peptide than non-growing stationary cells.

In order to know whether the level of ET-1 mRNA is different in various stages of cell cycle, we measured the level of mRNA of cells in S and non-S phases by \textit{in situ} hybridization\textsuperscript{15}. The analysis showed that cells in S phase express ET-1 mRNA higher than those in non-S phase. Furthermore, cells in both S and non-S phases increase the expression of ET-1 mRNA with aging. Thus, the data clearly indicate that an elevated expression of ET-1 mRNA in senescent cells is not due to the arrest in cell growth and suggest that there are, at least, two independent ways of increase in ET-1 gene expression: S phase and senescence. Several cell-cycle-dependent genes can be induced by the treatment of serum stimulation\textsuperscript{44}, but DNA synthesis in senescent cells is not initiated. This indicates that senescent cells are not equal to that of young growth-arrested quiescent cells. The increased expression of ET-1 gene in senescent cells might be brought by such a difference between young and senescent cells in regulation of gene expression. It is also known that the increase in expression of ET-1 gene is brought by shear stress, TGF-\(\beta\), TPA and insulin, and the specific sequences responsible for these responses are proved/suggested to exist on the up-stream region of ET-1 gene\textsuperscript{45–48}.

Fibronectin (FN) gene is up-regulated in both senescent fibroblasts and endothelial cells\textsuperscript{49,50}. Some extent of the up-regulation of FN gene has also been known in quiescent fibroblasts. The results that ET-1 gene is up-regulated in senescent cells but expressed low in young quiescent cells suggest that the ways of up-regulation of FN and ET-1 are different. On the contrary, the possibility remains that the way of up-regulation of ET-1 expression in senescent cells is the same as that of FN and only the way of cell cycle-dependent regulation is different from each other. Thus, it is important to elucidate the ways of the modulation of gene expression on both ET-1 and FN to understand the mechanism of cellular aging. There is a report indicating that ET-1 can up-regulate expression of FN gene\textsuperscript{51}. This suggests that the increased expression of ET-1 is the primary effect of cellular senescence and the enhanced expression of FN might be a secondary effect of that in endothelial cells. However, it may not be the case in fibroblasts since ET-1 peptide is not detectable in fibroblasts. Further studies are necessary to understand the mechanism of cellular aging.

Endothelin-1 production by cultured cardiac myocytes

It has been reported that preproET-1 mRNA was detected in heart tissues by Northern blot analysis\textsuperscript{52}, but ET-1 peptide has not been detected in the normal heart tissues. A heart consists of myocytes and non-myocytes such as fibroblasts, vascular endothelial and smooth muscle cells, etc. In culture of these cells, we showed positive immunological staining by ET-1 antibody only in the cardiac myocytes\textsuperscript{52}. To detect the ET-1 mRNA expression, reverse transcription-polymerase chain reaction (RT-PCR) using the specific primers of ET-1 was performed for freshly isolated and cultured cardiac myocytes\textsuperscript{52}. Southern blot hybridization under a stringent condition of the PCR product using the human ET-1 cDNA as a probe proved that both of freshly isolated and cultured cardiac myocytes express ET-1 mRNA. About 40%
stronger signal was observed in the RT-PCR product from cultured cardiac myocytes than that from freshly isolated cells. Thus, there was the possibility that cultured neonatal rat ventricular cardiac myocytes expressed preproET-1 mRNA as well as vascular endothelial cells. As PCR is highly sensitive, there is a possibility that even a very small amount of remaining non-myocytes, especially vascular endothelial cells, in the myocyte preparation can lead the result of detection of ET-1 mRNA. However, at the culture condition, the cardiac myocytes were stained very well by antibody to ET-1. To further confirm that the cultured cardiac myocytes secreted ET-1 into the culture medium, the level of ET-1 peptide in the culture medium of myocytes or non-myocytes was measured by radio-immunoassay. Immunologically reactive ET-1 was detected in the culture medium of cardiac myocytes, but it was not detected in the culture medium of non-myocytes.

As described above, the cardiac myocytes were stained very well by antibody to ET-1 at the culture condition. On the contrary, heart tissues from neonatal rats were not stained by the same antibody. This indicated that the introduction of myocytes into the culture condition induced ET-1 synthesis in the cardiac myocytes. Furthermore, it is possible that some kind of stimulation (humoral or physical) to hearts promotes the production of ET-1 in the cardiac myocytes in vivo.

It has been reported that ET-1 has positive inotropic effect on heart, stimulates the secretion of atrial natriuretic peptide (ANP), a vasodilatation factor, in rat atrial myocytes. These reports suggest that ET-1 acts on hearts as compensatory manner against to the reduced blood flow resulted from vascular contraction caused by ET-1 itself. However, where the ET-1 comes from and affects to the heart in vivo are not known. The amount of ET-1 in the culture medium of the purified cardiac myocytes was about 9.5 pM. Previously, we have reported that ET-1 induced hypertrophy and contractility of neonatal rat cardiac myocytes in vitro, and the effective doses of ET-1 were above 100 pM. Therefore, the level of ET-1 peptide in the culture medium of cardiac myocytes is not effective for the stimulation of the cultured cardiac myocytes. On the other hand, ET-1 levels in the blood were also reported in the patients with pulmonary hypertension and cardiogenic shock, and the concentrations of ET-1 reported were below few pM. Therefore, it is questionable whether this very low plasma concentration would have a significant influence on vessel tone. However, they have speculated in the report that ET-1 is released not only (or even predominantly) toward the lumen, but also in the abluminal direction. If ET-1 is, similarly, secreted by cardiac myocytes and affects to their neighbor cells (paracrine) or to themselves (autocrine), the local level of ET-1 may be enough to stimulate the myocytes.

Endothelin production under high oxygen condition

Heart is one of the largest oxygen-demanding organs in the body. However, heart cell cultures are ordinarily undertaken at 5% CO₂-air atmosphere in a culture apparatus. The cells utilize oxygen from culture medium, and the amount of oxygen in the medium depends on the diffusion coefficient of oxygen to the media at 37°C. However, in the pharmacological studies, a papillary muscle dissected from heart requires the 95% O₂-5% CO₂ gas supply to mimic in vivo responses to physical or chemical stimulators. Therefore, we considered that the ordinary oxygen concentration of atmosphere (21%) for the culture of cardiac myocytes may be rather lower than in vivo for the cells. We undertook the culture of the cardiac myocytes under the atmosphere of 50% O₂-5% CO₂-air as a high oxygen culture. The results showed that immunologically reactive ET-1 level in the culture medium at 50% oxygen culture was 1.3 pM. On the contrary, though the immunologically reactive ET-1 level at 21% oxygen culture was not different with that of the high oxygen culture during the first 24 hrs incubation period, it increased to 4.2 pM during 48 hrs incubation period. It is well known that high oxygen cul-
tures are harmful to many types of cells by the production of oxygen radicals. However, our condition of the high oxygen culture of the cardiac myocytes did not damage the myocytes.

At the myocyte culture of 21% oxygen concentration atmosphere, protein synthesis was stimulated by addition of ET-1 in a dose-dependent manner. However, ET-1 did not stimulate the protein synthesis when the cells were cultured at 50% oxygen concentration atmosphere. ET-1 production at the ordinary culture was about three times larger than that at the high oxygen culture. This indicates that the ET-1 production ability was influenced by the oxygen concentration in the cultures. If the oxygen concentration of the ordinary culture atmosphere is considered as a hypoxic condition for the cardiac myocytes, the decrease of oxygen supply to the myocytes may lead cells to synthesize and secrete ET-1. This is consistent with the report that ET-1 synthesis is stimulated under the condition of hypoxia in cultured human endothelium.

ET-1 binding assay was carried out. The data indicated that the ET-1 binds to a single class of binding sites in the cardiac myocytes under the conditions both of 50% and the ordinary oxygen cultures. The number of ET-1 binding site at 21% oxygen culture was 60% larger than that at 50% oxygen culture. It has been reported that ET-1 binding sites in cardiac myocytes increased at myocardial ischemia, re-perfusion, and re-oxygenation of hearts in vivo. Our results in together with these reports suggest that low oxygen increases the ET-1 binding site in the cardiac myocytes. Furthermore, there is a possibility that ET-1 may be produced and act to maintain the beating ability at the emergency such as decrease or lack of oxygen supply to the cardiac myocytes. At the same time, the cells also prepare the ET receptors under such hypoxic conditions. However, long-term deficiency of oxygen supply may cause irreversible damage to the cardiac cells.

Saito et al. showed in a recently paper that fibroblasts cultured in low oxygen condition are extended their life span in comparison with those in the ordinal 20% oxygen condition. It is known that high oxygen is harmful for many types of cells and the 20% oxygen atmosphere seems the case on the life span of fibroblasts. On the contrary, the 21% oxygen atmosphere seems to be uncomfortable for cardiac myocytes and the 50% oxygen may be comfortable. Thus, oxygen concentration of atmosphere in culture seems to be a very important factor for the study of gene expression and in vitro life span of cells. There is a report that ET-1 synthesis is stimulated under the condition of hypoxia in cultured human endothelium. We showed here that ET-1 synthesis is higher in senescent endothelial cells and reported previously that protein synthesis of neonatal rat cardiac myocytes was stimulated by addition of ET-1 to the culture in a dose-dependent manner and that ET-1 induced hypertrophy and contractility of the myocytes. Therefore, increased ET-1 synthesis in the elderly may lead stimulation of protein synthesis of cardiac myocytes and further hypertrophy. Hypertrophy may also be led by the state of less oxygen supply which stimulates ET-1 synthesis of endothelial cell (and cardiac myocyte itself). However, ET-1 synthesized by endothelium is diluted immediately by the blood and, thus, the concentration of ET-1 may be too low to stimulate the protein synthesis of myocytes because the effective doses of ET-1 were above 100 pM in vitro. Indeed, ET-1 levels reported in the blood of patients with pulmonary hypertension were below few pM.

We showed that the smooth muscle cell itself synthesized and secreted ET-1. This suggests the possibility of autocrine action of ET-1 on smooth muscle cells. On the other hand, ET-1 receptor exists on the surface of endothelial cell itself, suggesting the possibility of cross-talk between endothelial cells and smooth muscle cells. Similar possibility can be in heart: ET-1 stimulates the rhythmic heart-beat of cardiac myocytes and is produced by the cardiac myocyte itself under the condition of hypoxia. It is interesting how these findings relate to the physiological and pathological phenomena.
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