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Epidermal growth factor (EGF) decreased endothelin-2 (ET-2) production in human renal adenocarcinoma cells

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Production of immunoreactive (ir-) endothelin-2 (ET-2) in renal adenocarcinoma cells, ACHN, was reduced by transforming growth factor-β, basic fibroblast growth factor, transforming growth factor-α and, most strikingly, by epidermal growth factor (EGF). These growth factors did not show such inhibitory effects on the secretion of ir-ET-1 in ET-1-producing cells, indicating that the production of ET-2 and ET-1 is regulated differently by the growth factors. EGF specifically reduced the secretion of not only ir-ET-2 but also ir-big ET-2 with only a small decrease in total protein synthesis. Northern blot analysis indicated that EGF controls the ET-2-production at the transcription levels of ET-2 gene.

Endothelin-2; Epidermal growth factor; Enzyme immunoassay; ACHN cell

1. INTRODUCTION

Endothelins (ETs), namely ET-1, ET-2, and ET-3, are a recently discovered family of peptides consisting of 21 amino acid residues and two disulfide bonds [1,2]. Among them, ET-1, originally identified as an endothelium-derived contracting factor, has attracted much attention from the viewpoint of its potent and long-lasting vasoconstriction and pressor activity. ET-1 showed various pharmacological activities in both cardiovascular and non-cardiovascular tissues [3]. Especially, ET-1 is thought to play important roles in the kidney in the control of mesangial cell function, glomerular filtration rate, renal vascular tone [4] and ion transport/water permeability [5].

ET-2 differs from ET-1 in two amino acid residues in humans and is characterized as the most hydrophobic endothelin [2]. Immunoreactive (ir-) ET-2 was found in the intestine and colon in rats [6]. In humans, renal medulla is also an organ expressing high levels of mRNAs of prepro-ET-2, as well as prepro-ET-1 [7]. Since ET-2 is as potent as ET-1 in vasoconstricting activity, and shares two classes of receptors with ET-1 [8.9], it is of particular interest to examine how the functions and production of these endothelins are regulated, especially in the kidney. However, there have been few reports concerning physiologic and pathophysiologic roles of ET-2 and regulation of its synthesis.

Recently, a human cell line derived from renal adenocarcinoma, ACHN, was found to produce a large amount of ET-2 but not ET-1 [10]. The discovery of the ET-2-producing cells enabled us to isolate cDNAs coding for human prepro-ET-2 [11] and investigate the regulation of ET-2 biosynthesis.

It has been reported that the production of ir-ET-1 is enhanced by transforming growth factor (TGF)-β in PAE (porcine aortic endothelial cells) [1,12] and HepG-2 (human hepatocellular carcinoma cells) [12]. In the present study, we investigated the effects of various growth factors on the secretion of ET-2 in ACHN, and compared those with the secretion of ET-1 in MDCK (canine normal kidney cells) [12,13], HepG-2 and PAE. These studies showed that epidermal growth factor (EGF), a growth factor expressed abundantly in the kidney, markedly inhibited ET-2 production in ACHN.

2. MATERIALS AND METHODS

2.1. *Materials*

Basic-fibroblast growth factor (bFGF), EGF, insulin, nerve growth factor (NGF), TGF-α and TGF-β were obtained from Wako Pure Chem. (Osaka, Japan); insulin-like growth factor(IGF)-I and platelet-derived growth factor (PDGF) from Boehringer (Mannheim, Germany); IGF-II from Bachem Inc. (Torrance, USA); bovine serum albumin (BSA) from Sigma Chem. Co. (St. Louis, USA); ET-1, ET-2 and big ET-2 from Peptide Institute (Osaka, Japan); [^{3}H]leucine from Amersham (Buckinghamshire, England).

Abbreviations: ET-1, ET-2 and ET-3, endothelin-1, -2 and -3; EIA, enzyme immunoassay; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; IGF-I and IGF-II, insulin-like growth factor-I and -II; NGF, nerve growth factor; PDGF, platelet-derived growth factor; TGF-α and -β, transforming growth factor-α and -β.

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2.2. *Cells and culture conditions*

Ir-ET-1 or ir-ET-2 secreted in PAE, MDCK, HepG-2 or ACHN were previously characterized [10,12,13]. PAE was prepared according to the methods of Neich et al. [14]. ACHN, MDCK and HepG-2 were obtained from American Type Culture Collection. PAE was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (50 μg/ml) [12]. ACHN, MDCK, and HepG-2 were cultured in minimum essential medium supplemented with non-essential amino acids (MEM-NEAA), FCS and antibiotics, as given above [12].

The cells were grown to confluency in 24-well plates and washed with 1 ml of serum-free DMEM. Then, the medium was replaced with DMEM containing various factors to be tested and 0.1% of BSA. After incubation for 24 h, the culture medium was harvested and subjected to the EIAs.

2.3. *Detection of ET-1, ET-2 and big ET-2*

A sandwich-type EIA for ET-1 [13,15] was used to measure ET-1 and ET-2 in the culture medium. The EIA detects ET-2 as well as ET-1 (detection limit, 0.2 pg/well) without the crossreaction with ET-3 and big ETs. A sandwich-EIA for big ET-2 consists of two monoclonal antibodies, AwETN40 [15] and bET-20 [16], directed against the N-terminal portion of ET-2 and C-terminal portion of big ET-2(22–37), respectively. The EIA specifically detects as little as 0.4 pg/well of big ET-2 [16]. These EIAs were carried out in the same manner described previously [15].

2.4. *[^{3}H]leucine incorporation*

ACHN cells were grown confluently in 96-well plates and the culture medium was replaced with 200 μl of fresh serum-free MEM-NEAA containing 5 and 20 ng/ml of EGF. After 3 h exposure of EGF to the cells, the medium was replaced either with leucine-free RPMI-1640 containing the same concentrations of EGF and 0.25 μCi/well of [^{3}H]leucine or with fresh serum free MEM-NEAA containing the same concentrations of EGF. After another incubation for 3 h, the cells incubated in the medium containing [^{3}H]leucine were collected on the filter and their radioactivity was measured with a scintillation counter (LS 5801, Beckman Instruments Inc., Palo Alto, CA). Similarly, the culture media of the cells incubated in the fresh serum-free MEM-NEAA were subjected to the EIA for ET-2.

2.5. *Northern blot analysis*

ACHN cells were pre-incubated for 1 day with serum-free MEM containing 0.2% BSA and then incubated for 4 h in MEM with or without 20 ng/ml EGF. A poly(A)$^+$ RNA was prepared using mRNA isolation kit (Invitrogen, San Diego, CA). Northern blot analysis was carried out according to the methods of Thomas et al. [17]. A 10 μg portion of poly(A)$^+$ RNA separated by electrophoresis on 1.2% agarose gel was transferred to the nitrocellulose filter (Schleicher & Schnell, Dassel, Germany) and hybridized in the buffer containing 50% formamide at 42°C for 16 h with ^{32}P-labelled human ET-2 cDNA, pH ET-2 (k), cloned in advance from ACHN [11]. After hybridization, the filter was washed with 2×SSC (0.3 M NaCl, 30 mM trisodium citrate) and 0.1% SDS at 52°C.

3. RESULTS

3.1. *Effects of various factors on the secretion of ir-ET-1 and ir-ET-2*

ACHN, MDCK, HepG-2 and PAE were used to investigate the regulatory effects of various growth factors on the secretion of ir-ET-1 or ir-ET-2 (Table I). The basal level of ir-ET-2 in the culture medium of ACHN was approximately 400 pg/10^6 cells/day and those of ir-ET-1 in the culture media of PAE, MDCK and HepG-2 were 2600, 350 and 50 pg/10^6 cells/day, respectively.

Secretion of ir-ET-2 in ACHN was inhibited by TGF-β, bFGF, TGF-α and, most strikingly, by EGF at concentrations less than 10^{-8} M, whereas insulin, IGF-I, IGF-II, PDGF and NGF did not show any inhibitory effects on the secretion of ir-ET-2 in ACHN at concentrations of 10^{-8} M or more. Secretion of ir-ET-1 was enhanced by various growth factors in HepG-2, and slightly enhanced by TGF-β and PDGF in MDCK and PAE. EGF (3×10^{-9} M, 20 ng/ml) only slightly affected the production of ir-ET-1 in MDCK and PAE. bFGF and TGF-α have a tendency similar to EGF in the regulation of the synthesis of ET-1 and ET-2.

3.2. *Effects of EGF on the secretion of ir-ET-2 and ir-big ET-2 in ACHN*

Fig. 1 shows the time-course of the ir-ET-2 release into the medium. The inhibition of ir-ET-2 production was significant ($P<0.001$) at 5 h after the addition of

Table I

Effects of various growth factors on the secretion of ir-ETs in ACHN, MDCK, HepG-2 and PAE

Samples	Concentration (M)	ir-ET-2[1]	ir-ET-1[1]		
		ACHN (%)[2]	MDCK (%)	HepG-2 (%)	PAE (%)
Control		100	100	100	100
EGF	3×10^{-9}	15	87	220	110
TGF-α	4×10^{-9}	21	89	290	nt[3]
TGF-β	2×10^{-10}	46	140	670	150
bFGF	3×10^{-10}	22	81	150	110
Insulin	1×10^{-6}	130	110	nt	88
IGF-I	1×10^{-7}	140	100	nt	100
IGF-II	1×10^{-7}	95	86	nt	79
PDGF	1×10^{-8}	110	190	330	140
NGF	1×10^{-8}	110	130	180	79

[1] Ir-ETs in the culture medium were measured by a sandwich-EIA for ET-1 fully crossreactive with ET-2.
[2] Results are expressed in % of control.
[3] not tested.

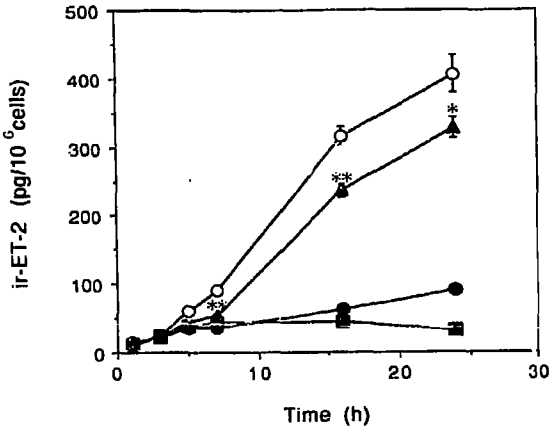

Fig. 1. Time-course of the inhibiting effects of EGF on the ir-ET-2 secretion in ACHN. The ACHN cells were cultured without (○) or with EGF (▲, 0.2 ng/ml; ●, 2 ng/ml; ■, 20 ng/ml) and aliquots of the culture medium were subjected to the sandwich-EIA for ET-1(ET-2). Each point represents the mean ± SEM (n=6). Asterisks show statistically significant differences from control (*P < 0.05, **P < 0.001).

EGF at the concentration of 0.2 ng/ml. EGF at the concentrations 2 and 20 ng/ml almost completely inhibited the secretion of ir-ET-2.

By the use of an EIA for big ET-2, it was found that ACHN secreted 200 pg/10^6 cells/day of ir-big ET-2 concomitantly with ir-ET-2. As shown in Fig. 2, EGF reduced the secretion of ir-big ET-2 along with ir-ET-2 in a dose- and time-dependent manner. The addition of 2 and 20 ng/ml of EGF similarly blocked the secretion of ir-big ET-2 in ACHN.

3.3. *Effects of EGF on the protein synthesis in ACHN*

[^{3}H]Leucine incorporation into ACHN was investigated in the presence and absence of EGF (Fig. 3). After 3 h exposure to 5 and 20 ng/ml of EGF, the ir-ET-2

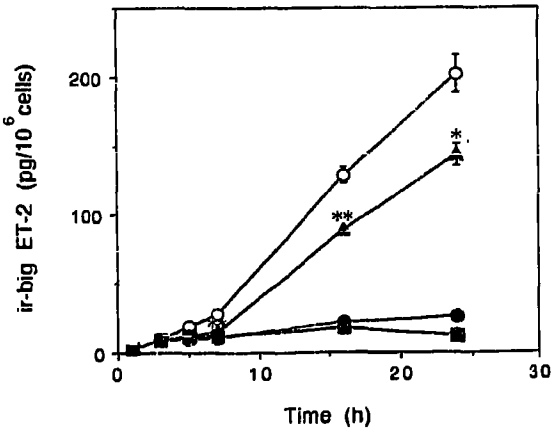

Fig. 2. Time-course of the inhibiting effects of EGF on the ir-big ET-2 secretion. The ACHN cells were cultured without (○) or with EGF (▲, 0.2 ng/ml; ●, 2 ng/ml; ■, 20 ng/ml). The culture medium was assayed for ir-big ET-2 by the sandwich-EIA. Asterisks show statistically significant differences from control (*P < 0.005, **P < 0.001). Details are shown in Fig. 1.

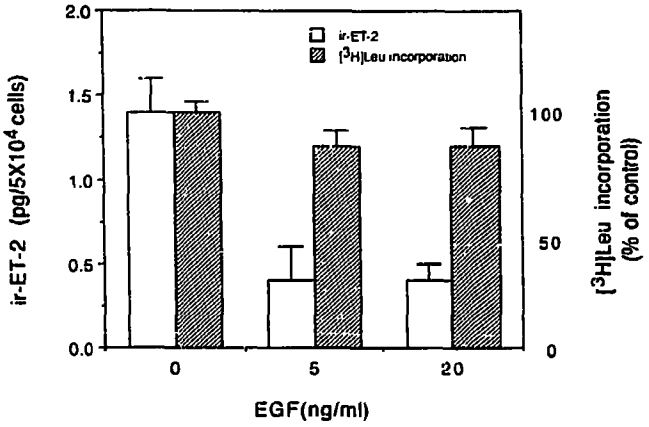

Fig. 3. Effect of EGF on the [^{3}H]leucine incorporation (hatched bars) and secretion of ir-ET-2 (open bars) in ACHN cells. Each point represents the mean ± SEM (n=6).

production during another 3 h incubation was reduced to 20–25% of the basal level with only a small decrease in the incorporation of [^{3}H]leucine into the cells. These results indicate that the inhibition of ir-ET-2 secretion by EGF was not due to the decrease in the total protein synthesis.

3.4. *Effects of EGF on ET-2 gene transcription in ACHN*

To examine the effects of EGF on the expression levels of the ET-2 gene, poly(A)$^+$ RNA derived from ACHN cells cultured with or without EGF was analyzed by Northern blotting using ^{32}P-labelled human ET-2 cDNA as a probe. As shown in Fig. 4, a single band corresponding to the ET-2 mRNA was observed at the position of 1.4 kbase. The band was obscured in the

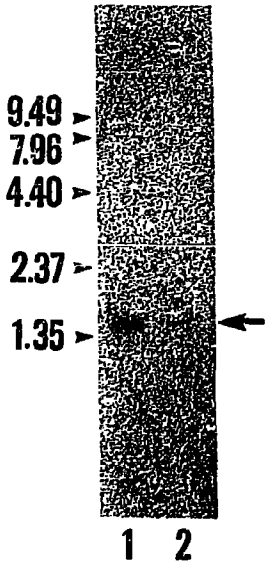

Fig. 4. Northern blot analysis of the poly(A)$^+$ RNA derived from ACHN cells cultured for 4 h with (lane 2) or without (lane 1) 20 ng/ml EGF. The arrow indicates the size of ET-2 gene transcripts (1.4 kb). Molecular size markers are shown on the left.

experiment using RNA of the cells treated with 20 ng/ml of EGF, indicating that EGF decreased the transcription of ET-2 gene.

4. DISCUSSION

The present study has shown that the production of ET-1 and ET-2 is regulated differently by various growth factors. TGF-β enhanced the secretion of ir-ET-1 in MDCK and other cells as described previously [1,12], but reduced ir-ET-2 secretion in ACHN, indicating that the production of ET-1 and ET-2 is regulated in the opposite direction by TGF-β. EGF strongly inhibited ET-2 secretion in ACHN, but only slightly affected the ir-ET-1 secretion in MDCK and endothelial cells, and enhanced ir-ET-1 secretion in HepG-2. TGF-α, another ligand for the EGF receptor [18], and bFGF also markedly decreased ir-ET-2 secretion in ACHN. The reduction of the ET-2 secretion by EGF was observed with only a small decrease in total protein synthesis, indicating that the reduction is specific to ET-2. Northern blot analysis showed that EGF controlled the ET-2 production at the transcription levels of prepro-ET-2 gene. This result was consistent with the fact that big ET-2 levels also decreased along with those of ET-2.

EGF positively and negatively regulates the transcription of various genes. Enhancement of the transcription of proto-oncogenes such as c-jun, c-fos and c-myc was observed in the mitogenic stimulation of cultured rat and mouse fibroblasts by EGF [19,20]. On the other hand, EGF decreases mRNA levels of inhibin in cultured rat granulosa cells [21]. Similar to the observation that the ET-2 secretion was inhibited by bFGF, the transcription of the inhibin gene was also repressed by bFGF [21]. Since the EGF and bFGF receptors have cytoplasmic tyrosine domains [22,23], signal transductions triggered by tyrosine kinase may participate in the regulation of the transcriptions of ET-2 and inhibin genes by EGF, TGF-α and bFGF.

The renal medulla is an organ expressing relatively high levels of prepro-ET-2 mRNA in humans [7]. In addition, ACHN was established from human renal adenocarcinoma which arises from the renal tubular epithelial cells [24]. Although the localization of ET-2 in the kidney has not yet been clarified in detail, the close relation of ET-2 with the renal tissues is also suggested by the fact that a relatively high amount of ir-big ET-2 but not ir-ET-1 or ir-big-ET-1 is found in human urine compared to plasma (unpublished observation).

Various growth factors have been thought to play roles in the kidney [25]. Although EGF is detectable in many adult mammalian tissues such as mammary and salivary glands, one major site of production is the kidney [26,27]. In humans, intense immunostaining of EGF was observed in the renal medulla [27]. EGF is present abundantly in the kidney as the large precursor protein [26], which is thought to retain EGF-like biological activity [28]. Therefore, it may be possible that EGF or a large precursor form of EGF represses transcription of prepro-ET-2 gene at a physiological condition in the kidney. In addition, concerning pathological roles of EGF in the kidney, it was reported that renal prepro-EGF mRNA and urinary EGF secretion were reduced during cysplatin and ischemia-induced acute renal failures in rats [29]. Interestingly, ET(s) appeared to be a deleterious mediator in drug- or ischemia-induced renal failures in rats [30,31]. Therefore, the inhibitory mechanism of the ET-2 gene expression by EGF might play a role in the prevention of overproduction of the potentially toxic peptide, ET-2, in the physiological conditions of the kidney.

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