主論文 1

Brain Natriuretic Peptide Is Present in the Human Amniotic Fluid and Is Secreted from Amnion Cells*

HIROAKI ITOH, NORIMASA SAGAWA, MASAAKI HASEGAWA, ATSUHIKO OKAGAKI, KUMIKO INAMORI, YOSHIYUKI IHARA, TAKAHIDE MORI, YOSHIHIRO OGAWA, SHIN-ICHI SUGA, MASASHI MUKOYAMA, KAZUWA NAKAO, AND HIROO IMURA

Department of Gynecology and Obstetrics, Second Division Department of Medicine (Y.O., S.-i.S., M.M., K.N., H.I.), Kyoto University Faculty of Medicine, Kyoto 606, Japan

ABSTRACT

The presence and biochemical characteristics of human brain natriuretic peptide (hBNP) in the amniotic fluid at various gestational ages were investigated. The hBNP-like immunoreactivity (hBNP-LI) levels in amniotic fluid, determined by RIA, were 118.7 ± 57.6 pmol/L (mean $\pm$ SEM; n = 5) and 107.7 ± 8.7 pmol/L (n = 9) in the first and second trimesters of pregnancy, respectively; it was significantly decreased to 28.4 ± 5.1 pmol/L (n = 9) in the third trimester. However, human atrial natriuretic peptide-like immunoreactivity (hANP-LI) was not detected (<0.3 pmol/L) in any of these samples. Northern blot analysis demonstrated hBNP mRNA in human amnion tissue. Moreover, cul-

tured amnion cells secreted a significant amount of hBNP-LI ($100–200$ fmol/10^6 cells/day), but not hANP-LI, into the culture medium. The synthesis of hBNP in cultured amnion cells was further confirmed using the polymerase chain reaction. High performance gel permeation chromatography of hBNP-LI extracted from human amniotic fluid and the culture medium of amnion cells revealed that the predominant molecular form of hBNP-LI in both samples was the hBNP precursor, with an approximate mol wt of 12 kilodaltons.

These findings indicate that hBNP is present in the human amniotic fluid, and that amnion cells synthesize hBNP and secrete it into the amniotic cavity. (*J Clin Endocrinol Metab* **76**: 907–911, 1993)

BRAIN natriuretic peptide (BNP), a novel natriuretic, diuretic, and smooth muscle relaxant peptide originally isolated from the porcine brain, has a structure highly homologous to that of atrial natriuretic peptide (ANP), and has biological functions similar to those of ANP (1, 2). We previously reported that BNP is synthesized in the porcine and rat heart and is secreted into the circulation as a cardiac hormone (3, 4). Recently, we isolated human BNP (hBNP) from human atrium and established a specific RIA for hBNP using a monoclonal antibody against hBNP (2, 5, 6). We subsequently revealed that hBNP is mainly secreted from the ventricle and that the synthesis and secretion of hBNP in the heart are increased in patients with cardiovascular diseases, such as congestive heart failure (5, 6).

During normal and pathological pregnancies, significant hemodynamic alteration occurs, with drastic changes in the levels of various vasoactive hormones (7). Among these hormones, ANP is reported to be involved in the homeostasis of maternal and fetal circulations (8, 9).

We previously demonstrated the presence of ANP in the rat brain (10), and Gardner *et al.* (11) detected ANP mRNA in extracardiac tissues, such as rat lung and pituitary gland. Moreover, ANP may be related to hormone secretion, such as aldosterone secretion from adrenal gland and vasopressin

secretion from the pituitary (12–14). Recently, it was reported that hANP was involved in water and sodium absorption across the intestine (15, 16) and that BNP stimulated phosphate secretion from porcine colon epithelium (17). However, no information is available on the implication of natriuretic peptides in the regulation of amniotic fluid volume, which is essential for the fetus to survive.

In the present study, to investigate the presence of natriuretic peptides in the amniotic cavity, we measured hBNP-like immunoreactivity (hBNP-LI) and hANP-like immunoreactivity (hANP-LI) in the amniotic fluid at various gestational ages by RIA. We also measured the secretion of hBNP-LI from amnion cells in primary culture and demonstrated the gene expression of hBNP in amnion tissue and cultured amnion cells by Northern blot analysis and polymerase chain reaction, respectively.

Materials and Methods

Collection of amniotic fluid

Amniotic fluid samples were collected after receiving informed consent at the time of therapeutic abortion, diagnostic amniocentesis, and cesarean section at various gestational ages. None of the women had complications of hypertension or cardiovascular diseases. Samples were immediately transferred into chilled siliconized glass tubes containing aprotinin (1000 kallikrein inhibitor units/mL) and EDTA disodium salt (1 mg/mL), mixed gently, and centrifuged at $1500 \times g$ for 20 min at 4 C. The supernatant was stored at -20 C until assay.

Collection and preparation of tissue

Amnion, chorion laeve, decidua vera tissue, and chorion plate were separated from placentae obtained by normal vaginal delivery at term

and by abortions in the first and second trimesters (12, 13, 14, 20, and 23 weeks gestation). Human ventricular tissue was obtained from patients without cardiovascular complications at the time of autopsy for pathological examination. These samples were immediately frozen in liquid nitrogen and stored at −70 C until the extraction of BNP or total RNA.

Extraction of BNP from amnion tissue was carried out as described previously (6). This extract was used for the RIA of BNP-LI in amnion tissues.

Culture of human amnion cells

A primary culture of human amnion cells was conducted according to the method described by Okita et al. (18). After filtration of the dispersed amnion cells through 2-mm and 300-μm mesh, amnion cells in the filtrate were resuspended at a concentration of 2×10^5 cells/mL in Dulbecco's Minimum Essential Medium containing 10% fetal calf serum and seeded on a gelatin-coated dish. The incubation was conducted under the atmosphere of 5% CO_2 in air at 37 C. Every 2 days, an aliquot of the medium was sampled and stored at −20 C until assay for hBNP-LI and hANP-LI, then the medium was changed to fresh Dulbecco's Minimum Essential Medium containing 10% fetal calf serum. The purity of cultured amnion cells was examined by immunohistochemical staining for cytokeratin, vimentin, and CA19-9 (19, 20). The pattern of immunohistochemical staining for these substances in the cultured amnion cells was the same as that observed in the amnion epithelium in situ (19, 20).

RIA for hBNP and hANP

The RIA for hBNP was performed using a specific monoclonal antibody (KY-hBNP-I) to hBNP, as described previously (5, 6). In some samples, hBNP-LI was extracted with a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA). The cross-reactivity of this RIA to hANP was less than 0.005% on a molar basis. The minimum detectable quantity was 0.3 fmol/tube, and the 50% binding intercept was 3 fmol/tube. The RIA for hANP was performed as previously described (21). The cross-reactivity of RIA for hANP to hBNP was less than 0.01% on a molar basis. hBNP and hANP were purchased from Peptide Institute, Inc. (Minoh, Japan). All other reagents used were of analytical grade.

High performance gel permeation chromatography (HP-GPC)

The extracts, obtained with a Sep-Pak C_{18} cartridge from amniotic fluid or culture medium of amnion cells, were subjected to HP-GPC on a TSK-GEL G2,000 SW column (7.5 × 600 mm; Toyo Soda, Tokyo, Japan). The column was eluted with 10 mM trifluoroacetic acid containing 0.3 M sodium chloride and 30% acetonitrile as a solvent (6, 22).

Northern blot analysis of hBNP mRNA and hANP mRNA

Total RNA was extracted from tissues in 4 M guanidium thiocyanate buffer. Northern blot analysis was carried out using the hBNP and hANP cDNA probes, as previously described (23–25). β-Actin mRNA levels were demonstrated to be equivalent among different RNA samples, using human β-actin genomic probe (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Polymerase chain reaction (PCR)

To confirm that BNP is actually synthesized in cultured amnion cells, the PCR was carried out. Total RNA was extracted from cultured amnion cells at the time they reached confluence and 10 days after reaching confluence as well as from the ventricular tissue of the heart, as described above. After reverse transcription of 5 μg of these total RNA by oligo(dT) priming and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratory, Gaithersberg, MD), the resulting single stranded cDNA was subjected to PCR using primers described previously (4, 6, 25).

Statistical analysis

Statistical analysis of the data was performed by Student's t test or analysis of variance where appropriate. All values were expressed as the mean ± SEM. The number of observations is indicated in parentheses.

Results

hBNP-LI level in amniotic fluid

The dilution curve of amniotic fluid paralleled the standard curve of hBNP. hBNP-LI levels in the amniotic fluid of the first and second trimesters were 118.7 ± 57.6 pmol/L (mean ± SEM; n = 5) and 107.7 ± 8.7 pmol/L (n = 9), respectively. In the third trimester, the hBNP-LI level in the amniotic fluid was 28.4 ± 5.1 pmol/L (n = 9), which was significantly (P < 0.01) lower than those in the first and second trimesters. In contrast, hANP-LI was not detected (<0.3 pmol/L) in any amniotic fluid samples examined.

hBNP-LI level in the amnion tissue and synthesis of hBNP in amnion tissue

To investigate the source of hBNP in the amniotic fluid, we measured hBNP-LI levels in the extracts from amnion tissues. The hBNP-LI level in the amnion tissue was 262 ± 59.5 fmol/g tissue (mean ± SEM; n = 3) in the second trimester. However, the hBNP-LI level in the amnion tissue at term was less than 90 fmol/g tissue (the lower limit of detectability) in all three samples examined. On the other hand, hANP-LI levels in the extract from the same amnion tissues were less than 90 fmol/g tissue (the lower limit of detectability) in both the second trimester and at term. To confirm further the synthesis of hBNP in amnion tissue, we measured the hBNP mRNA level in amnion tissues in the second trimester and at term, chorionic plate at term, chorion laeve at term, and decidua vera in the second trimester and at term. In amnion tissue in the second trimester, Northern blot analysis using the hBNP cDNA probe identified a single mRNA band of the same size as the hBNP mRNA from the human ventricular tissue (Fig. 1). However, no hybridizing band of hBNP mRNA was detected in the other tissues examined. By contrast, no hybridizing signal of hANP mRNA was detected in any of the tissues examined.
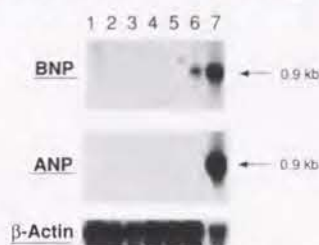

FIG. 1. Detection of hBNP mRNA in the human amnion tissue by Northern blot analysis. Northern blot analysis of hBNP mRNA and hANP mRNA from decidua tissue at term (lane 1) and in the second trimester (lane 2), placenta at term (lane 3), chorion laeve at term (lane 4), amnion tissue at term (lane 5) and in the second trimester (lane 6), and heart (lane 7) was performed as described in *Materials and Methods*. Twenty micrograms of total RNA were used in each lane. The relative amount of β-Actin mRNA in each sample was determined by rehybridization of the plate. Kb, Kilobases.

Synthesis and secretion of hBNP by cultured amnion cells

The dilution curve of culture medium of amnion cells also paralleled the standard curve of hBNP. To investigate the synthesis and secretion of hBNP from amnion cells, we measured hBNP-LI levels in the culture medium of amnion cells. The hBNP-LI level in the culture medium remained low until the amnion cells reached confluence. However, the hBNP-LI level increased several days after reaching confluence. Ten days later, the hBNP-LI level in the culture medium after 48 h of incubation was 151.9 ± 18.8 pmol/L (mean ± SEM; n = 5). From the changes in the hBNP-LI level in the culture medium, the rate of hBNP synthesis by cultured amnion cells was calculated. The rate of hBNP synthesis on the tenth day of confluence was 188.9 ± 24.9 fmol/10^6 cells·day (n = 5, Fig. 2). By contrast, hANP-LI was not detected (<0.3 pmol/L) in the culture medium or amniotic fluid.

To further confirm the synthesis of hBNP in the primary culture of amnion cells, PCR was carried out. The PCR successfully amplified a single size fragment of the predicted size (462 basepairs), visualized by ethidium bromide staining, using cDNAs from amnion cells 10 days after reaching confluence as well as those from heart, while no amplification was observed using cDNAs from amnion cells at the time they reached confluence (Fig. 3).
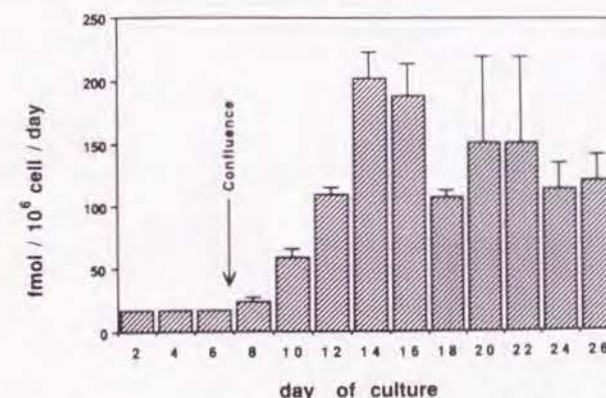

FIG. 2. Time course of hBNP-LI secretion from amnion cells in culture. Amnion cells reached confluence on the seventh day of culture. *Hatched columns and vertical bars* represent the mean and SEM of hBNP-LI secreted per 10^6 cells/day in five dishes.
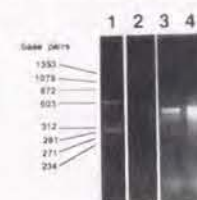

FIG. 3. Detection of hBNP mRNA in the cultured amnion cells by DNA PCR. The total RNA extracted from cultured amnion cells at the time they reached confluence (lane 2) and 10 days after reaching confluence (lane 3) and from heart (lane 4) were used. After reverse transcription of 5 μg total RNA, the resulting cDNA was subjected to PCR, and the PCR product was fractionated on a 1.4% agarose gel and visualized by ethidium bromide staining. The positions of size markers ($\phi x/HaeIII$; lane 1) are shown on the *left*.

Molecular form of hBNP-LI in amniotic fluid and culture medium of amnion cells

The molecular form of hBNP-LI in the extracts from the second trimester amniotic fluid and the culture medium of amnion cells was analyzed by HP-GPC. As shown in Fig. 4, hBNP-LI in both samples was eluted in a single peak, with an approximate mol wt of 12 kilodaltons, corresponding to the hBNP precursor. A similar pattern was observed when the same samples were assayed using another anti-hBNP antibody with different specificities (data not shown).

Discussion

The present study demonstrated that hBNP is present in human amniotic fluid, while the hANP-LI level was below assay sensitivity. The hBNP-LI level in amniotic fluid was much higher (10- to 100-fold) than those in third trimester maternal plasma and umbilical venous plasma (~2–3 pmol/L) (26, 27). The hBNP-LI level in the first urine of newborns
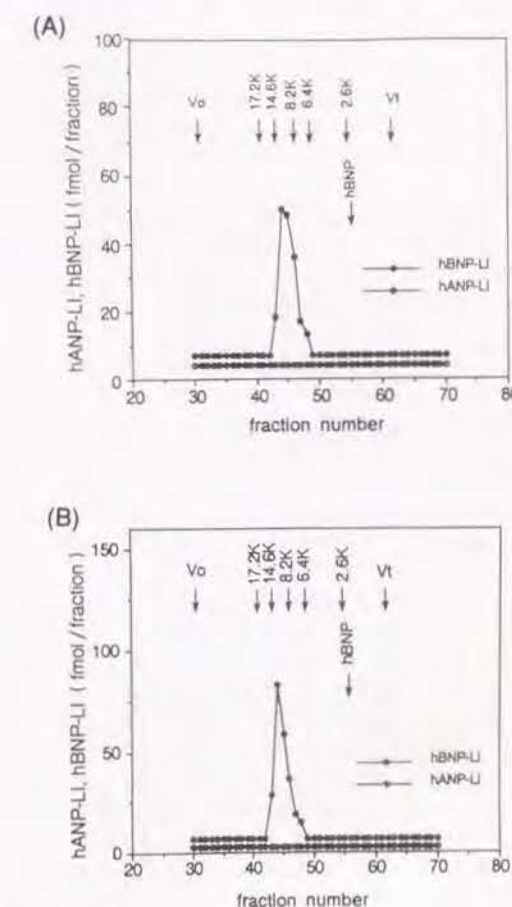

FIG. 4. Typical HP-GPC profiles of the hBNP-LI extracted from the amniotic fluid and culture medium of amnion cells. A, Extracts from amniotic fluid of normal pregnancy in the second trimester. B, Extracts from the supernatant of primary culture of amnion cells. ●, hBNP-LI in each fraction of HP-GPC; O, hANP-LI. In both extracts, hANP-LI was below the assay sensitivity in all fractions. The flow rate was 0.3 mL/min, and the fraction volume was 0.36 mL. Vo, Void volume; Vt, total volume.

was approximately 0.7 pmol/L, much lower than that in the amniotic fluid (Itoh, H., N. Sagawa, and K. Nakao, unpublished finding). Moreover, the predominant molecular form of hBNP-LI in the amniotic fluid, the hBNP precursor with an approximate mol wt of 12 kilodaltons, was quite different from the molecular form of hBNP-LI in adult plasma (mainly 3 kilodaltons) (6). These findings suggest that such a high hBNP-LI level in the amniotic fluid does not originate from maternal plasma or fetal urine. The secretion of hBNP from the cultured amnion cells indicates that hBNP-LI in the amniotic fluid originates from the amnion cells. The synthesis of hBNP in amnion tissue and cultured amnion cells was confirmed by Northern blot analysis and the PCR technique. This is the first report on BNP gene expression in tissues other than heart (25) and brain (1). By contrast, hANP mRNA was not detected by this method in any samples examined.

In the present study, hBNP mRNA was detected only in amnion tissue in the second trimester and in cultured amnion cells 10 days after reaching confluence, but not in amnion tissue at term or in amnion cells at the time they reached confluence. The regulatory mechanism of hBNP gene expression in amnion cells is not clear at present. The amnion tissue is avascular tissue and relatively free from the circulating hormones. Therefore, investigation of the synthesis and secretion of the hBNP in cultured amnion cells may provide additional information on the regulation of hBNP synthesis and secretion.

It is not known whether hBNP synthesized in the amnion cells can permeate fetal membranes into the neighboring tissues, such as decidua, placenta, or myometrium. In our preliminary experiment, a high hBNP-LI level was found in the fluid of extraembryonic coelom in two cases of first trimester abortion (338.2 and 79.1 pmol/L). These levels were similar to those in the amniotic fluid of the same patients. This finding may indicate that the hBNP precursor synthesized in amnion cells can permeate the amniotic membrane and reach the tissues neighboring the amniotic cavity, such as chorion, decidua, placenta, and myometrium. Although it is not known whether the hBNP precursor binds to natriuretic peptide receptors in these tissues, natriuretic peptide receptors are present in placenta (28, 29). Moreover, in our preliminary experiment, the natriuretic peptide receptors were identified in decidua vera tissue (Itoh, H., N. Sagawa, and K. Nakao, unpublished observation). Taken together these findings lead us to speculate that the large amount of hBNP secreted from amnion cells exerts biological activity on tissues surrounding the amniotic cavity. For example, hBNP secreted from amnion cells may regulate amniotic fluid volume by modulating the transport of electrolytes and water across the fetal membranes. Another possibility is that hBNP may reach the decidua and myometrium and play some role in the prevention of initiation of preterm labor by relaxing the uterine myometrium until term, when the hBNP-LI level in amniotic fluid is significantly decreased. Further investigation on the characterization of natriuretic peptide receptors in the tissues of amnion, chorion laeve, decidua vera, myometrium, and placenta, as well as the biological activity of the hBNP precursor should provide

more information on the biological significance of hBNP in human amniotic fluid.

Finally, this primary culture system of amnion cells may serve as a useful model in investigating the regulation of the synthesis and secretion of hBNP, since amnion cells secrete only hBNP, in contrast to cardiocytes, which secrete both hANP and hBNP.

References

1. Sudoh T, Kangawa K, Minamino N, Matsuo H. 1988 A new natriuretic peptide in porcine brain. Nature (Lond). 332:78–81.
2. Kambayashi Y, Nakao K, Mukoyama M, et al. 1990 Isolation and sequence determination of human brain natriuretic peptide in human atrium. FEBS Lett. 259:341–345.
3. Saito Y, Nakao K, Itoh H, et al. 1989 Brain natriuretic peptide is a novel cardiac hormone. Biochem Biophys Res Commun. 158:360–368.
4. Ogawa Y, Nakao K, Mukoyama M, et al. 1991 Natriuretic peptides as cardiac hormones in normotensive and spontaneously hypertensive rats: the ventricle is a major site of synthesis and secretion of brain natriuretic peptide. Circ Res. 69:491–500.
5. Mukoyama M, Nakao K, Saito Y, et al. 1990 Human brain natriuretic peptide, a novel cardiac hormone. Lancet. 335:801–802.
6. Mukoyama M, Nakao K, Hosoda H, et al. 1991 Brain natriuretic peptide as a novel cardiac hormone in humans—evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. J Clin Invest. 87:1402–1412.
7. Weiner CP, Robillard JE. 1988 Atrial natriuretic factor, digoxin-like immunoreactive substance, norepinephrine, epinephrine, and plasma renin activity in human fetuses and their alteration by fetal disease. Am J Obstet Gynecol. 159:1353–1360.
8. Hirai N, Yanaihara T, Nakayama T, Ishibashi M, Yamaji T. 1988 Plasma levels of atrial natriuretic peptide during normal pregnancy and in pregnancy complicated by hypertension. Am J Obstet Gynecol. 159:27–31.
9. Kikuchi K, Nakao K, Hayashi K, et al. 1987 Ontogeny of atrial natriuretic polypeptide in the human heart. Acta Endocrinol (Copenh). 115:211–217.
10. Morii N, Nakao K, Sugawara A, et al. 1985 Occurrence of atrial natriuretic polypeptide in brain. Biochem Biophys Res Commun. 127:413–419.
11. Gardner DG, Deschepper CF, Ganong WF, et al. 1986 Extra-atrial expression of the gene for atrial natriuretic factor. Proc Natl Acad Sci USA. 83:6697–6701.
12. Atarashi K, Mulrow PJ, Franco-Saenz R, Snajdar R, Rapp J. 1984 Inhibition of aldosterone production by an atrial extract. Science. 224:992–994.
13. Matsuoka H, Fukui K, Dan Y, et al. 1991 Atrial natriuretic peptide inhibits the aldosterone response to metoclopramide in patients with glomerular disease and essential hypertension. Clin Exp Pharmacol Physiol. 18:8:557–562.
14. Samson WK. 1985 Atrial natriuretic factor inhibits dehydration and hemorrhage-induced vasopressin release. Neuroendocrinology. 40:277–279.
15. Kanai Y, Ohnuma N, Matsuo H. 1987 Rat atrial natriuretic polypeptide increases net water, sodium and chloride absorption across rat small intestine *in vivo.* Jpn J Pharmacol. 45:7–13.
16. Matsushita K, Nishida Y, Hosomi H, Tanaka S. 1991 Effects of atrial natriuretic peptide on water and NaCl absorption across the intestine. Am J Physiol. 260:R6–R12.
17. Traynor TR, O'Grady SM. 1991 Brain natriuretic peptide stimulates K and Cl secretion across porcine distal colon epithelium. Am J Physiol. 260:C750–C755.
18. Okita JR, Sagawa N, Casey ML, Snyder JM. 1983 A comparison of human amnion tissue and amnion cells in primary culture by morphological and biochemical criteria. In Vitro. 19:117–126.
19. Regauer S, Franke WW, Virtanen I. 1985 Intermediate filament cytoskeleton of amnion epithelium and cultured amnion epithelial cells: expression of epithelial cytokeratins in cells of a simple epithelium. J Cell Biol. 100:997–1009.
20. Kobayashi F, Sagawa N, Nanbu Y, et al. 1989 Immunohistochemical localization and tissue levels of tumor-associated glycoproteins CA125 and CA19-9 in the decidua and fetal membranes at various gestational ages. Am J Obstet Gynecol. 160:1232–1238.
21. Nakao K, Sugawara A, Morii N, et al. 1984 Radioimmunoassay for α-human and rat atrial natriuretic polypeptide. Biochem Biophys Res Commun. 124:815–821.
22. Ogawa Y, Nakao K, Mukoyama M, et al. 1990 Rat brain natriuretic peptide. Tissue distribution and molecular form. Endocrinology. 126:2225–2227.
23. Saito Y, Nakao K, Arai H, et al. 1989 Augmented expression of atrial natriuretic polypeptide gene in ventricle of human failing heart. J Clin Invest. 83:298–305.
24. Arai H, Nakao K, Saito Y, et al. 1988 Augmented expression of atrial natriuretic polypeptide gene in ventricles of spontaneously hypertensive rats (SHR) and SHR-stroke prone. Circ Res. 62:926–930.
25. Hosoda K, Nakao K, Mukoyama M, et al. 1991 Expression of brain natriuretic peptide gene in human heart. Production in the ventricle. Hypertension. 17:1152–1156.
26. Sagawa N, Itoh H, Hasegawa M, et al. 1991 Plasma levels of natriuretic peptides (ANP, BNP) in pregnancy-induced hypertension [Abstract]. J Perinat Med. 19(Suppl 2):74.
27. Itoh H, Sagawa N, Hasegawa M, et al. 1991 Concentrations of natriuretic peptides (ANP, BNP) in the umbilical cord blood and in the amniotic fluid [Abstract]. J Perinat Med. 19(Suppl 2):117.
28. Sen I. 1986 Identification and solubilization of atrial natriuretic factor receptors in human placenta. Biochem Biophys Res Commun. 135:480–486.
29. Salas SP, Power RF, Singleton A, Wharton J, Polak JM, Brown J. 1991 Heterogenous binding sites for alpha-atrial natriuretic peptide in human umbilical cord and placenta. Am J Physiol. 261:R633–R638.

主論文 2

Transforming Growth Factor-β Stimulates, and Glucocorticoids and Epidermal Growth Factor Inhibit Brain Natriuretic Peptide Secretion from Cultured Human Amnion Cells*

HIROAKI ITOH, NORIMASA SAGAWA, MASAAKI HASEGAWA, KUMIKO INAMORI,
HIROHISA UEDA, KYOKO KITAGAWA, HIDETAKA NANNO, YOSHIYUKI IHARA,
FUMINORI KOBAYASHI, TAKAHIDE MORI, SHIN-ICHI SUGA,
TAKAAKI YOSHIMASA, HIROSHI ITOH, AND KAZUWA NAKAO

Department of Gynecology and Obstetrics and Second Division Department of Medicine (S.-I.S., T.Y., Hiros.I., K.N.), Kyoto University Faculty of Medicine, Kyoto 606, Japan

ABSTRACT

We previously reported the massive secretion of brain natriuretic peptide (BNP) from human amnion cells and suggested the possible role of BNP in the maintenance of human pregnancy. In this study, to elucidate the regulatory mechanism of BNP secretion from amnion cells, we measured the BNP level in the culture medium of amnion cells by RIA after incubation in the presence of various substances.

Among the agents examined, cortisol (1×10^{-7} to 1×10^{-6} mol/L), dexamethasone (1×10^{-8} to 1×10^{-6} mol/L), and epidermal growth factor (EGF; 2×10^{-11} to 2×10^{-8} mol/L) inhibited BNP secretion from the cultured amnion cells in a dose-dependent manner. By con-

trast, transforming growth factor-β (TGFβ; 4×10^{-11} to 4×10^{-9} mol/L) caused a 3- to 5-fold increase in BNP secretion. TGFβ-augmented BNP secretion was abolished by the addition of cortisol or EGF to the culture medium.

Moreover, in this study, we revealed the presence of bioactive TGFβ in human amniotic fluid (~4×10^{-10} mol/L). The present finding of tight regulation of BNP secretion from amnion cells by cortisol, EGF and TGFβ, all at the concentrations physiologically present in human amniotic fluid, implies a physiological role of BNP secretion from amnion cells in the pregnant uterus. (*J Clin Endocrinol Metab* **79:** 176–182, 1994)

WE PREVIOUSLY found that brain natriuretic peptide (BNP), a second natriuretic peptide with natriuretic, diuretic, and vasodilative properties (1), was secreted from the porcine and rat heart as a cardiac hormone (2, 3). We subsequently isolated human BNP from human atrium and established a specific RIA for human BNP (4–6). Using this specific RIA, we have demonstrated that BNP is predominantly synthesized in and secreted from the ventricle, and that the plasma concentration of BNP as well as that of atrial natriuretic peptide (ANP) are significantly increased in patients with cardiovascular diseases (5, 6). These findings suggest the importance of BNP as a cardiac hormone. Recently, we found that the plasma BNP level is markedly elevated in women with pregnancy-induced hypertension (7) and in the umbilical vein of newborns with fetal distress (8), suggesting the pathophysiological importance of BNP in the maternal and fetal circulations during pregnancy.

Subsequently, we found that a high level (~110 pmol/L) of BNP, 50-fold higher than that in maternal or fetal plasma, is present in human amniotic fluid in the first and second trimesters of pregnancy, whereas the ANP level is below

assay sensitivity (<0.3 pmol/L) (9). The BNP level in amniotic fluid decreases in the third trimester of pregnancy to one fourth of that in the first and second trimesters (9). Northern blot analysis revealed that the BNP messenger ribonucleic acid was detected in amnion tissues during the second trimester, but was not detectable in the third trimester. Furthermore, human amnion cells in primary culture have been shown to secrete a large amount of BNP into the culture medium (9). Thus, we have concluded that the BNP in amniotic fluid mainly originates from amnion cells, and BNP synthesis in amnion cells decreases in the third trimester, suggesting the role of BNP in the maintenance of human pregnancy (9).

In this study, to elucidate the regulation of BNP secretion from amnion cells during pregnancy, we examined the effects of various substances, which are present in human amniotic fluid and postulated to be important for the maintenance of pregnancy, on BNP secretion from cultured human amnion cells. As we and others have demonstrated that human amniotic fluid contains endothelin-1 (ET-1), a potent vasoconstrictor, and ET-1 is secreted from cultured amnion cells (10–12), we simultaneously measured ET-1-like immunoreactivity (ET-1-LI) in the culture medium for comparison.

Materials and Methods

Reagent

Human BNP, ET-1, and ET-3 were obtained from Peptide Institute (Minoh, Japan). Epidermal growth factor (EGF) was kindly donated by

TABLE 1. Effects of various agents on BNP and ET-1 secretion from cultured human amnion cells

Agents	BNP secretion (fmol/10^6 cells · 72 h)	ET-1 secretion (fmol/10^6 cells · 72 h)
Exp 1		
Vehicle control	246.3 ± 29.9	126.0 ± 11.4
Estradiol (1 × 10^{-6} mol/L)	215.1 ± 11.5	103.9 ± 6.4
Progesterone (1 × 10^{-6} mol/L)	251.2 ± 25.7	103.5 ± 5.9
Exp 2		
Vehicle control	247.6 ± 20.0	163.0 ± 7.0
Norepinephrine (1 × 10^{-6} mol/L)	221.6 ± 41.6	150.0 ± 10.1
Isoproterenol (1 × 10^{-6} mol/L)	230.8 ± 27.8	150.1 ± 11.1
Dopamine (1 × 10^{-6} mol/L)	206.9 ± 19.5	152.7 ± 12.6
Exp 3		
Vehicle control	282.2 ± 53.3	116.0 ± 14.4
Dexamethasone (1 × 10^{-6} mol/L)	114.0 ± 33.9^a	132.0 ± 37.9
Cortisol (1 × 10^{-6} mol/L)	130.6 ± 19.7^b	120.4 ± 3.5
Exp 4		
Vehicle control	238.5 ± 63.2	ND
ET-1 (2 × 10^{-8} mol/L)	203.8 ± 59.5	ND
ET-3 (2 × 10^{-8} mol/L)	236.1 ± 23.4	ND
Exp 5		
Vehicle control	311.0 ± 25.1	198.0 ± 39.4
8-Bromo-cGMP (1 × 10^{-3} mol/L)	287.4 ± 17.2	227.7 ± 42.8
Exp 6		
Vehicle control	398.1 ± 17.5	89.9 ± 5.8
EGF (2 × 10^{-9} mol/L)	29.9 ± 2.5^b	252.2 ± 11.9^b
Exp 7		
Vehicle control	258.3 ± 42.6	178.7 ± 20.3
TGFβ (4 × 10^{-10} mol/L)	1047.3 ± 4.3^b	262.1 ± 3.2^a

Human amnion cells in primary monolayer culture were treated with various agents. After 72 h of incubation, culture media were sampled, and BNP and ET-1 concentrations were determined, as described in *Materials and Methods*. Data are expressed as the mean ± SEM of quadruplicate dishes. ND, Not determined.
^{a} $P < 0.01$ vs. vehicle controls.
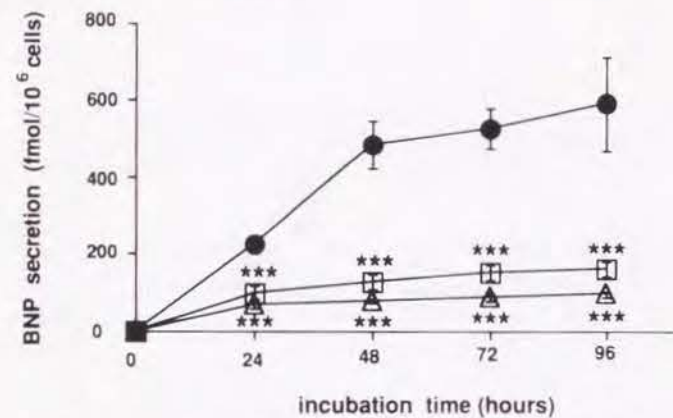^{b} $P < 0.001$ vs. vehicle controls.

incubation time (hours)

FIG. 1. Time course of BNP secretion from cultured amnion cells treated with dexamethasone and cortisol. Ten days after the cells reached confluence, dexamethasone and cortisol were added to the culture medium, and the cumulative amount of BNP secreted into the culture medium was determined by RIA. ●, Vehicle control; △, treatment with 1 × 10^{-6} mol/L dexamethasone; □, treatment with 1 × 10^{-6} mol/L cortisol. Values are the mean ± SEM of quadruplicate dishes. *Asterisks* indicate significant differences from the vehicle control values at the same incubation time. ★★★, $P < 0.001$. The time course of BNP secretion from amnion cells treated with dexamethasone or cortisol was significantly different from that in the vehicle control, as determined by repeated measures of ANOVA (ANOVA and Fisher's protected least significant difference test, $P < 0.001$ for both comparisons).

Ohtsuka Pharmaceutical Co. (Tokushima, Japan). Transforming growth factor-β1 (TGFβ1) and anti TGFβ1 rabbit sera were purchased from R & D Systems (Minneapolis, MN). All other reagents used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental protocol

A primary culture of human amnion cells was conducted as described previously (9, 13). The dispersed amnion cells were collected by centrifugation and resuspended in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal calf serum (FCS) and seeded 2 × 10^5 cells/well on a gelatin-coated 12-well plate. The incubation was conducted under an atmosphere of 5% CO$_2$ in air at 37 C. Every 2 days, the medium was replaced with fresh DMEM containing 10% FCS.

Ten days after the cells reached confluence, when BNP secretion from the amnion cells became stable (9), the medium was replaced with fresh DMEM containing 10% FCS, and the various substances listed in Table 1 were added to the culture medium. Dexamethasone, cortisol, estradiol, and progesterone were diluted with 100% ethanol, and the final concentration of ethanol in the culture medium was 0.1%. The same amount of ethanol was added to the vehicle control, and ethanol at this concentration had no effect on the BNP secretion from cultured amnion cells. Isoproterenol, norepinephrine, and dopamine were diluted with 1 mmol/L HCl and 0.1 mmol/L L-ascorbic acid, and the final concentrations of HCl and L-ascorbic acid in the culture medium were 100 and 10 nmol/L, respectively. ET-1 and ET-3 were diluted with 20 mmol/L potassium phosphate buffer containing 0.5% gelatin. TGFβ1 was diluted with 4 mmol/L HCl containing 0.1% BSA, and the final concentration of HCl in the culture medium was 100 nmol/L. The same amount of HCl was added to the vehicle control, and HCl at this concentration did not affect the pH of the medium (pH 7.5). 8-Bromo-cGMP was diluted with DMEM containing 10% FCS. Every 24 h, an aliquot of the medium was sampled and stored with aprotinin (1000 kallikrein inhibitor units/mL), Triton-X (0.5%), and EDTA disodium salt (1 mg/mL) at −20 C until assay. The amounts of BNP and ET-1 secreted from amnion cells were calculated from the changes in BNP and ET-1 levels in the culture medium, determined by RIA.
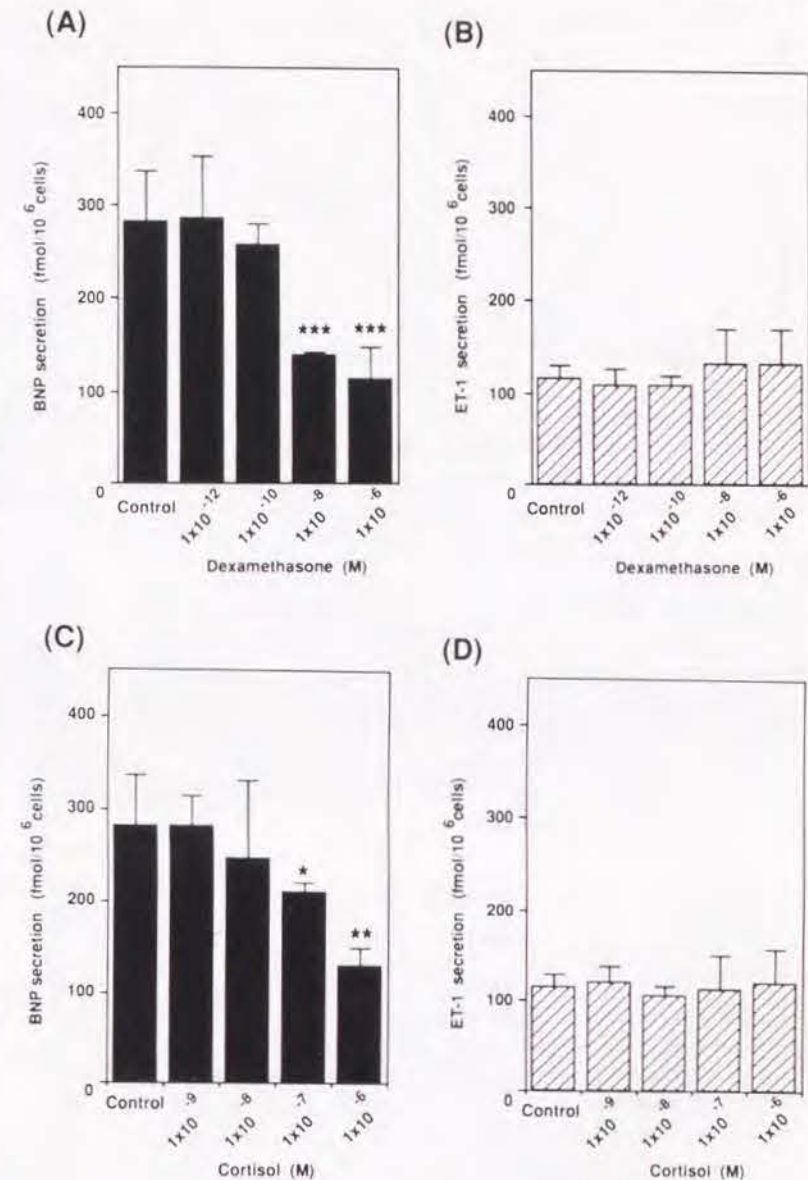
FIG. 2. Dose-dependent effects of dexamethasone and cortisol on the BNP and ET-1 secretion from cultured amnion cells. Ten days after the cells reached confluence, various concentrations of dexamethasone or cortisol were added to the culture medium. Then, the total amounts of BNP and ET-1 secreted from amnion cells into the culture medium during 72 h of incubation were determined by RIA. Values are the mean ± SEM of quadruplicate dishes. *Asterisks* indicate the significant differences from the vehicle-treated control values. ★, $P < 0.05$; ★★, $P < 0.01$; ★★★, $P < 0.001$. A and B, Effect of dexamethasone on BNP secretion (A) and ET-1 secretion (B). C and D, Effects of cortisol on BNP secretion (C) and ET-1 secretion (D).

RIA for human BNP and ET-1

The RIA for human BNP was performed using a specific monoclonal antibody (KY-hBNP-I) to human BNP, as described previously (6, 9). The cross-reactivities of this RIA to human ANP, C-type natriuretic peptide, and ET-1 were less than 0.005%, 0.5%, and 0.5% on a molar basis, respectively. The sensitivity and 50% inhibitory concentration of this assay were 0.3 and 3 fmol/tube, respectively.

The RIA for ET-1 was performed using a specific monoclonal antibody (KY-ET-1-IV) to ET-1, as described previously (10, 14). The cross-reactivities of this RIA to ET-2, ET-3, and big ET-1 were 80%, 20%, and 80% on a molar basis, respectively. The cross-reactivity of this RIA to human BNP was less than 0.1% on a molar basis. The sensitivity and 50% inhibitory concentration of this assay were 0.2 and 0.6 fmol/tube, respectively.

Bioassay for TGFβ

The bioassay of TGFβ was carried out as previously described (15, 16). In brief, CCL-64 mink lung epithelial cells (17) were inoculated at a density of 2 × 10^5 cells/well in 24-well plates 1 day before the assay,

using Minimum Essential Medium and 0.1 mmol/L nonessential amino acid (Life Technologies, Grand Island, NY) with 10% FCS. The subconfluent cells were washed and incubated with flesh DMEM containing 0.5% FCS and TGFβ1 (5–320 pmol/L), human amniotic fluid (2.5–20%) obtained from elective cesarean section cases at term or from diagnostic amniocentesis in the second trimester (16, 16, and 20 weeks of pregnancy), or conditioned medium from amnion cell culture. Twenty hours later, [^{3}H]thymidine (2 μCi/mL) was added to the medium, followed by further incubation for 20 h. Then, the amount of [^{3}H]thymidine incorporated was determined by a liquid scintillation counter. The growth inhibitory effect of TGFβ1 and amniotic fluid on CCL-64 was confirmed by blocking the growth inhibitory effect by a rabbit neutralizing anti-TGFβ1 antibody.

Analysis of data

Values are expressed as the mean ± SEM. All experiments were conducted in quadruplicate dishes. The experiments were repeated at least three times to confirm the results. Statistical analysis of the results was performed by Student's t test or analysis of variance (ANOVA), followed by Fisher's protected least significant difference test when the
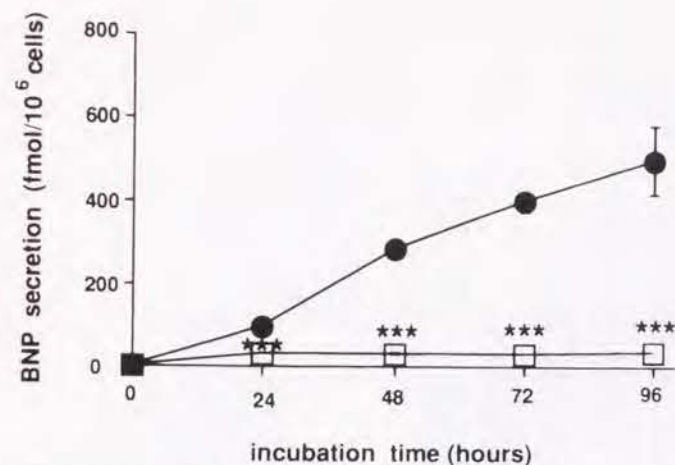
FIG. 3. Time course of BNP secretion from cultured amnion cells treated with EGF. Ten days after the cells reached confluence, 2×10^{-9} mol/L EGF were added to the culture medium, and the cumulative amount of BNP secreted into the culture medium was determined by RIA. ●, BNP secretion in the vehicle control; □, BNP secretion from amnion cells treated with EGF. Values are the mean ± SEM of quadruplicate dishes. Asterisks indicate significant differences from the vehicle control values at the same incubation time. ★★★, $P < 0.001$. The time course of BNP secretion from EGF-treated amnion cells was significantly different from that of the vehicle control by repeated measures of ANOVA (ANOVA and Fisher's protected least significant difference test, $P < 0.001$).

means of more than two groups were compared. The differences with $P < 0.05$ were regarded as significant.

Results

Effects of various agents on BNP and ET-1 secretion from cultured amnion cells

Table 1 summarizes the effects of various agents on BNP secretion from cultured amnion cells. Estradiol (1×10^{-6} mol/L); progesterone (1×10^{-6} mol/L); norepinephrine, isoproterenol, and dopamine (1×10^{-6} mol/L each); ET-1 ($2 \times$

10^{-8} mol/L); ET-3 (2×10^{-8} mol/L); and 8-bromo cGMP (1×10^{-3} mol/L) had no effect on BNP and ET-1 secretion.

On the other hand, dexamethasone (1×10^{-6} mol/L), cortisol (1×10^{-6} mol/L), and EGF (2×10^{-9} mol/L) significantly suppressed BNP secretion from cultured amnion cells. In contrast, TGFβ (4×10^{-10} mol/L) stimulated BNP secretion. In the same experiment, dexamethasone and cortisol did not affect ET-1 secretion. However, EGF and TGFβ significantly stimulated ET-1 secretion.

Effects of glucocorticoids on BNP secretion from cultured amnion cells

Figure 1 shows the time course of BNP secretion from cultured amnion cells in the absence or presence of glucocorticoids. BNP secretion from amnion cells was markedly suppressed by the addition of 1×10^{-6} mol/L dexamethasone to 29.1% and 11.0% of the vehicle control value at 24 and 96 h of incubation, respectively. As illustrated in Fig. 1, the addition of 1×10^{-6} mol/L cortisol also significantly suppressed BNP secretion from cultured amnion cells to 39.5% and 18.7% of the vehicle control value at 24 and 96 h of incubation, respectively.

Figure 2, A and C, shows the dose-dependent inhibition of BNP secretion by dexamethasone and cortisol, respectively. BNP secretion was inhibited 50% by dexamethasone and cortisol at 1.2×10^{-8} and 8.5×10^{-7} mol/L, respectively. On the other hand, ET-1 secretion from cultured amnion cells was not affected by the same treatments with dexamethasone or cortisol (Fig. 2, B and D).

Effects of EGF on BNP secretion from cultured amnion cells

As illustrated in Fig. 3, the addition of 2×10^{-9} mol/L EGF markedly suppressed BNP secretion from amnion cells (31.6% and 7.2% of the vehicle control value after 24 and 96 h of incubation, respectively). As shown in Fig. 4A, such an inhibition by EGF of BNP secretion from cultured amnion cells was dose dependent. BNP secretion was inhibited 50%
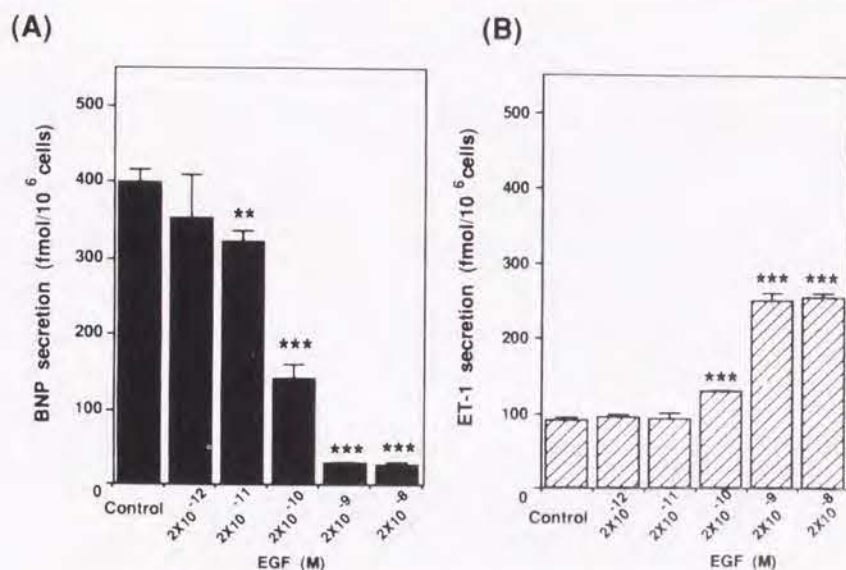

FIG. 4. Dose-dependent effects of EGF on BNP (A) and ET-1 (B) secretion from cultured amnion cells. Ten days after the cells reached confluence, various concentrations of EGF were added to the culture medium. Then, the total amounts of BNP and ET-1 secreted from amnion cells into the culture medium during 72 h of incubation were determined by RIA. Values are the mean ± SEM of quadruplicate dishes. Asterisks indicate significant differences from the vehicle control values. ★★, $P < 0.01$; ★★★, $P < 0.001$.
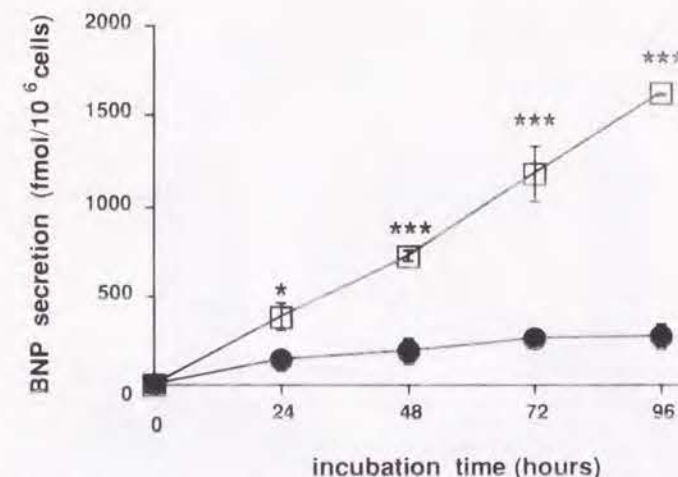
FIG. 5. Time course of BNP secretion from cultured amnion cells treated with TGFβ. Ten days after the cells reached confluence, 4×10^{-10} mol/L TGFβ were added to the culture medium, and the cumulative amount of BNP secreted into the culture medium was determined by RIA. ●, BNP secretion in the vehicle control; □, BNP secretion from amnion cells treated with 4×10^{-10} mol/L TGFβ. Values are the mean ± SEM of quadruplicate dishes. Asterisks indicate significant differences from the vehicle control values at the same incubation time. ★, $P < 0.05$; ★★★, $P < 0.001$. The time course of BNP secretion from TGFβ-treated amnion cells was significantly different from that in the vehicle control by repeated measures of ANOVA (ANOVA and Fisher's protected least significant difference test, $P < 0.001$).
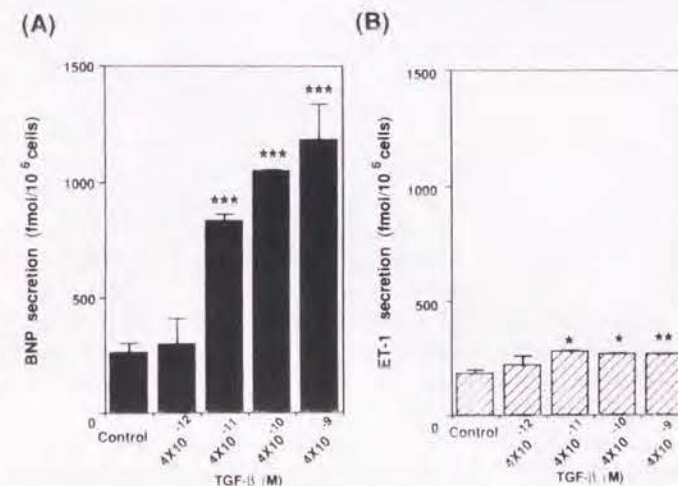

FIG. 6. Dose-dependent effects of TGFβ on BNP (A) and ET-1 (B) secretion from cultured amnion cells. Ten days after the cells reached confluence, various concentrations of EGF were added to the culture medium. Then, the total amounts of BNP and ET-1 secreted from amnion cells into culture medium during 72 h of incubation were determined by RIA. Values are the mean ± SEM of quadruplicate dishes. Asterisks indicate significant differences from the vehicle control values. ★, $P < 0.05$; ★★, $P < 0.01$; ★★★, $P < 0.001$.

by EGF at 1×10^{-10} mol/L.

In contrast, as shown in Fig. 4B, ET-1 secretion from amnion cells was enhanced dose-dependently by the addition of 2×10^{-10} to 2×10^{-8} mol/L EGF. ET-1 secretion was stimulated by 2×10^{-9} mol/L EGF to a 3-fold higher level than that of the vehicle control. The concentration of EGF

required for half-maximal stimulation was 3.5×10^{-10} mol/L.

Effects of TGFβ on BNP secretion from cultured amnion cells

Figure 5 shows the time course of BNP secretion from cultured amnion cells stimulated by 4×10^{-10} mol/L TGFβ. BNP secretion from cultured amnion cells during 96 h of incubation in the presence of 4×10^{-10} mol/L TGFβ was 5-fold higher than that in the vehicle control. As illustrated in Fig. 6A, this stimulation by TGFβ of BNP secretion during 72 h of incubation was dose dependent. The concentration of TGFβ required for half-maximal stimulation was 2.1×10^{-11} mol/L.

On the other hand, 4×10^{-11} to 4×10^{-9} mol/L TGFβ only slightly augmented ET-1 secretion from cultured amnion cells (Fig. 6B). The amount of ET-1 secreted in the presence of 4×10^{-9} mol/L EGF was 150% of that secreted in the vehicle control culture.

Effects of cortisol and EGF on TGFβ-induced BNP secretion from cultured amnion cells

The effects of cortisol and EGF on BNP secretion augmented by 4×10^{-10} mol/L TGFβ were examined. As shown in Fig. 7A, the augmentation of BNP secretion by TGFβ was dose dependently suppressed by cortisol. EGF also dose dependently suppressed TGFβ-stimulated BNP secretion (Fig. 7B).

Detection of biologically active TGFβ in human amniotic fluid

The growth-suppressive activity of TGFβ has been detected in the human amniotic fluid by a growth inhibition assay using CCL-64 mink lung epithelial cells. TGFβ concentrations in the amniotic fluid in the second trimester and at term were estimated to be 433 ± 54 pmol/L (mean ± SEM; n = 3) and 410 ± 137 pmol/L (n = 3), respectively. This growth-suppressive activity of amniotic fluid was blocked by a rabbit anti-TGFβ1 neutralizing antibody and was increased 4- to 5-fold by acid treatment of the amniotic fluid samples, indicating activation of the latent form of TGFβ by acid treatment (18) (data not shown). On the other hand, the growth-suppressive activity of TGFβ was not detected in the conditioned medium of amnion cell culture (<20 pmol/L).

Discussion

Among the agents examined in this study, dexamethasone, cortisol, and EGF suppressed BNP secretion from cultured amnion cells. By contrast, TGFβ augmented BNP secretion. The augmentation by TGFβ was blocked by simultaneous treatment with cortisol or EGF.

As cortisol is the most abundant glucocorticoid in the human amniotic fluid (19), we examined the effects of cortisol on BNP secretion from cultured amnion cells. Cortisol in human amniotic fluid originates from fetal urine, and the concentration of cortisol in the amniotic fluid increases during pregnancy from 1×10^{-8} mol/L in the second trimester
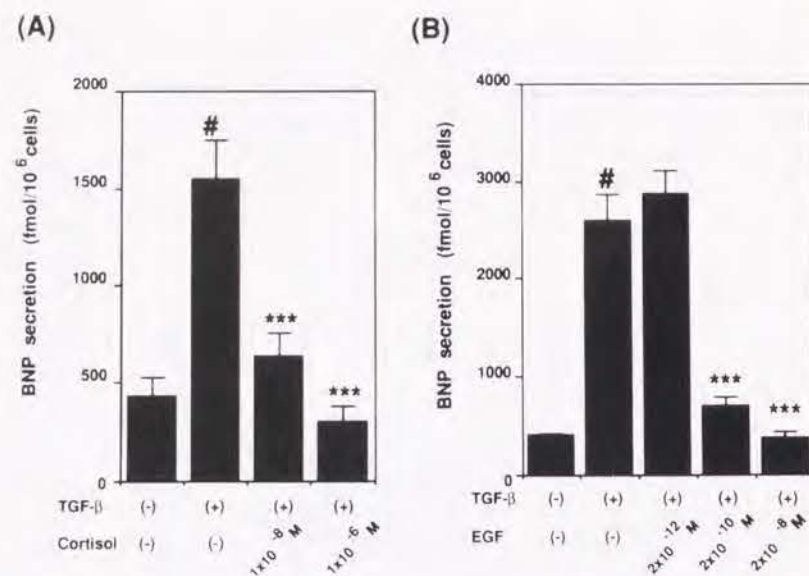
(A)   **(B)**

FIG. 7. Effects of cortisol (A) and EGF (B) on TGFβ-induced BNP secretion from cultured amnion cells. Ten days after the cells reached confluence, various amounts of cortisol or EGF were added to the culture medium together with 4×10^{-10} mol/L TGFβ. The total amounts of BNP and ET-1 secreted from amnion cells into culture medium during 72 h of incubation were determined by RIA. Values are the mean ± SEM of quadruplicate dishes. #, Significantly ($P < 0.001$) different from the vehicle control value. ★★★, Significantly ($P < 0.001$) different from the value with 4×10^{-10} mol/L TGFβ alone.

to 1×10^{-7} mol/L at term, reflecting the development of fetal adrenal gland (19, 20). In the present study, BNP secretion from cultured amnion cells was significantly suppressed by the addition of 1×10^{-7} mol/L cortisol, which resembles its physiological concentration in amniotic fluid at term. However, a lower concentration of cortisol (1×10^{-8} mol/L) did not suppress BNP secretion. Thus, the increased concentration of cortisol in the amniotic fluid, which originates from the mature fetal adrenal gland, might act on the amnion tissue and suppress BNP secretion from amnion tissue at term.

A large amount of EGF is present in human amniotic fluid at term (21, 22). EGF in amniotic fluid is postulated to be involved in the initiation of human labor by stimulating prostaglandin production in amnion cells (23, 24). In the present study, 2×10^{-11} to 2×10^{-8} mol/L EGF dose dependently inhibited BNP secretion from cultured amnion cells. The EGF level in human amniotic fluid increases gradually up to 2×10^{-10} mol/L at term (21, 22). Kniss *et al.* (24) reported that EGF in amniotic fluid is derived at least in part from fetal kidney. Thus, the mature fetus at term may secrete an increasing amount of EGF into amniotic fluid in the form of fetal urine, and the increased concentration of EGF might inhibit BNP secretion from amnion cells.

The present study demonstrated that the bioactive TGFβ (~400 pmol/L) exists in the amniotic fluid in the second trimester and at term. TGFβ at 4×10^{-10} mol/L, the same order as the physiological concentration in amniotic fluid, markedly enhanced BNP secretion from cultured amnion cells. In the present study, TGFβ was not detected in the conditioned medium of amnion cell culture, suggesting that TGFβ in amniotic fluid did not originate from amnion cells. Although the exact origin of TGFβ in the amniotic fluid has not been clarified, several investigators have reported gene expression of TGFβ in human placental trophoblast (25) and human decidua (26). Thus, we speculate that BNP secretion from amnion cells is regulated by TGFβ secreted from tissues surrounding the amnion tissue. Further investigation is re-

quired to test this interesting hypothesis.

TGFβ-augmented BNP secretion from cultured amnion cells was significantly suppressed by the addition of cortisol or EGF at concentrations physiologically present in amniotic fluid at term. The TGFβ concentration in amniotic fluid at term was similar to that during the second trimester. Therefore, these findings suggest that BNP secretion from amnion cells is augmented or maintained by TGFβ during the second trimester when the concentrations of EGF and cortisol are low, and that BNP secretion might be suppressed by the increased levels of cortisol and EGF at term (19–22).

Thus, the marked changes in the BNP concentration in amniotic fluid during pregnancy can be explained by the actions of TGFβ, EGF, and cortisol. However, there remains a possibility that some other factors in amniotic fluid may contribute to the regulation of BNP secretion from amnion cells. Another possibility that may affect BNP secretion from amnion cells is that these agents are metabolized *in vivo* by peptidases in the amnion cells, and their activities change depending on gestational age. Further studies are required to examine such a possibility.

Recently, Potvin and Varma (27) reported that ANP inhibited tension development by the uterus of 10- to 14-day pregnant rats. Our preliminary observation showed the gene expression of receptor for ANP and BNP (ANP-A receptor) in human decidua and uterine smooth muscle in the second trimester (Itoh, H., and N. Sagawa, unpublished findings). Moreover, our previous study demonstrated that BNP secreted from amnion cells may reach the tissues surrounding the amniotic cavity (9). Thus, BNP secreted from amnion cells might reach the tissues of decidua and myometrium and play some role in the maintenance of pregnancy, especially in the first and second trimesters, and the effect of BNP on these tissues may be attenuated at term when BNP secretion from amnion cells decreases markedly. Further studies on the biological activity of BNP on the tissues adjacent to the amniotic membrane, including decidua and

myometrium, are required to confirm the role of amniotic and culture media of human pregnancy.

References

1. **Sudoh T, Kangawa K, Minamino M, Matsuo H.** 1988 A new natriuretic peptide in porcine brain. Nature (Lond). 332:78–81.
2. **Saito Y, Nakao K, Itoh H, et al.** 1989 Brain natriuretic peptide is a novel cardiac hormone. Biochem Biophys Res Commun. 158:360–368.
3. **Ogawa Y, Nakao K, Mukoyama M, Hosoda K, et al.** 1991 Natriuretic peptide as cardiac hormones in normotensive and spontaneously hypertensive rats. Circ Res. 69:491–500.
4. **Kambayashi Y, Nakao K, Mukoyama M, et al.** 1990 Isolation and sequence determination of human brain natriuretic peptide in human atrium. FEBS Lett. 259:341–345.
5. **Mukoyama M, Nakao K, Saito Y, et al.** 1990 Human brain natriuretic peptide, a novel cardiac hormone. Lancet. 335:801–802.
6. **Mukoyama M, Nakao K, Hosoda K, et al.** 1991 Brain natriuretic peptide as a novel cardiac hormone in humans–evidence for an exquisite dual natriuretic peptide system atrial natriuretic peptide and brain natriuretic peptide. J Clin Invest. 87:1402–1412.
7. **Itoh H, Sagawa N, Mori T, Mukoyama M, Nakao K, Imura H.** 1993 Plasma brain natriuretic peptide level in pregnant women with pregnancy-induced hypertension. Obstet Gynecol. 82:71–77.
8. **Itoh H, Sagawa N, Hasegawa M, et al.** 1993 Brain natriuretic peptide level in the umbilical venous plasma are elevated in fetal distress. Biol Neonate. 64:18–25.
9. **Itoh H, Sagawa N, Hasegawa M, et al.** 1993 Brain natriuretic peptide is present in the human amniotic fluid and is secreted from amnion cells. J Clin Endocrinol Metab. 76:907–911.
10. **Hasegawa M, Sagawa N, Ihara Y, et al.** 1991 Concentration of endothelin-1 in human amniotic fluid at various stages of pregnancy. J Cardiovasc Pharmacol. 17(Suppl):S440–S442.
11. **Casey ML, Word RA, MacDonald PC.** 1991 Endothelin-1 gene expression and regulation of endothelin mRNA and protein biosynthesis in avascular human amnion–potential source of amniotic fluid endothelin. J Biol Chem. 266:5762–5768.
12. **Mitchell MD, Lundin-Schiller S, Edwin SS.** 1991 Endothelin production by amnion and its regulation by cytokines. Am J Obstet Gynecol. 165:120–124.
13. **Okita JR, Sagawa N, Casey ML, Snyder JM.** 1983 A comparison of human amnion tissue and amnion cells in primary culture by morphological and biochemical criteria. In Vitro. 19:117–126.
14. **Saito Y, Nakao K, Itoh H, et al.** 1989 Endothelin in human plasma and culture media of aortic endothelial cells–detection and characterization with radioimmunoassay using monoclonal antibody. Biochem Biophys Res Commun. 161:320–326.
15. **Itoh H, Mukoyama M, Pratt RE, Gibbons GH, Dzau VJ.** 1993 Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. J Clin Invest. 91:2268–2274.
16. **Altman DJ, Schneider SL, Thompson DA, Cheng HL, Tomasi TB.** 1990 A transforming growth factor β2 (TGFβ2)-like immunosuppressive factor in amniotic fluid and localization of TGFβ2 mRNA in the pregnant uterus. J Exp Med. 172:1391–1401.
17. **Danielpour D, Dart LL, Flanders KC, Roberts AB, Sporn MB.** 1989 Immunodetection and quantitation of two forms of transforming growth factor-beta (TGFβ1 and TGFβ2) secreted by cells in culture. J Cell Physiol. 138:79–86.
18. **Miyazono K, Hellman U, Wernstedt C, Heldin CH.** 1988 Latent high molecular weight complex of transforming growth factor β1. Purification from human platelets and structural characterization. J Biol Chem. 263:6407–6415.
19. **Fencl MD, Tulchinsky D.** 1975 Total cortisol in amniotic fluid and fetal lung maturation. N Engl J Med. 292:133–136.
20. **Murphy BEP, Patrick J, Denton RL.** 1975 Cortisol in amniotic fluid during human gestation. J Clin Endocrinol Metab. 40:164–167.
21. **Hofmann GE, Abramowicz JS.** 1990 Epidermal growth factor (EGF) concentrations in amniotic fluid and maternal urine during pregnancy. Acta Obstet Gynecol Scand. 69:217–221.
22. **Goodyer PR, Fata J, Mulligan L, et al.** 1991 Expression of transforming growth factor-α and epidermal growth factor receptor in human fetal kidneys. Mol Cell Endocrinol. 77:199–206.
23. **Casey ML, Korte K, MacDonald PC.** 1988 Epidermal growth factor stimulation of prostaglandin E2 biosynthesis in amnion cells–induction of prostaglandin H2 synthase. J Biol Chem. 263:7846–7854.
24. **Kniss DA, Mershon J, Su HC, et al.** 1990 Evidence of a role for protein kinase C in epidermal growth factor-induced prostaglandin E2 synthesis in amnion cells. Am J Obstet Gynecol. 163:1883–1890.
25. **Dungy LJ, Siddiqi TA, Khan S.** 1991 Transforming growth factor-β1 expression during placental development. Am J Obstet Gynecol. 165:853–857.
26. **Kauma S, Matt D, Strom S, Eierman D, Turner T.** 1990 Interleukin-1β, human leukocyte antigen HLA-DRα, and transforming growth factor-β expression in endometrium, placenta, and placental membranes. Am J Obstet Gynecol. 163:1430–1437.
27. **Potvin W, Varma DR.** 1990 Refractoriness of the gravid rat uterus to tocolytic and biochemical effects of atrial natriuretic peptide. Br J Pharmacol. 100:341–347.