Localization of clearance receptor in rat lung and trachea: association with chondrogenic differentiation

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Fujishige, Kotomi, Noriyuki Yanaka, Hiroyuki Akatsuka, and Kenji Omori. Localization of clearance receptor in rat lung and trachea: association with chondrogenic differentiation. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L425–L431, 1998.—The lung is rich in atrial natriuretic peptide binding sites, and the majority of them are considered to be the natriuretic peptide clearance receptor (NPR-C). In this study, localization of NPR-C in the rat lung and trachea was investigated by immunohistochemical analysis with the specific antibody. Positive staining was observed in the epithelial cell layers of the trachea and bronchiole and the myocardium surrounding the pulmonary vein. Moreover, expression of NPR-C was seen in mesenchymal cells; it was especially strong in cells in the perichondrium and decreased in chondrocytes in the cartilage. Because mesenchymal cells in the perichondrium differentiate to chondrocytes, NPR-C expression is suggested to be associated with chondrogenic differentiation. The chondrogenic cell line ATDC5 was used to study NPR-C expression during chondrogenic differentiation in vitro. The undifferentiated ATDC5 cells expressed NPR-C at a much higher level than the differentiated ATDC5 cells, in accordance with the observation of the immunohistochemical analysis in the cartilage. These findings suggest that NPR-C expression is differentially regulated in chondrocytes and that the natriuretic peptides may play a role in regulating chondrocyte development in the lung.

immunohistochemical analysis; natriuretic peptide receptor; ATDC5 cells

THE NATRIURETIC PEPTIDES are recognized to be a family of at least three polypeptide hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide, and C-type natriuretic peptide (CNP) (7, 32). ANP, in particular, has been shown to play a number of roles including diuresis, natriuresis, vasorelaxation, and inhibition of aldosterone release (6, 9, 20, 26). Recently, natriuretic peptides have also been expected to be involved in bone formation. That is, guanosine 3′,5′-cyclic monophosphate (cGMP) produced in response to ANP and CNP inhibited the proliferation and promoted the differentiation of osteoblast-like cells from newborn rat calvaria (11). CNP was also a stimulator of the differentiation of clonal osteoblastic MC3T3-E1 cells (16). The biological effects of natriuretic peptides are mediated through membrane-bound receptors [natriuretic peptide receptor (NPR)-A, NPR-B, and NPR-C]. NPR-A and NPR-B are glycoproteins of ̴ 120 kDa with guanylate cyclases coupled to the production of cGMP (5, 29), whereas NPR-C, a homodimer of ̴ 70 kDa, has a shorter intracellular tail lacking the cyclase domain and is therefore uncoupled from cGMP (8, 24). NPR-C has a very high affinity for all members of the natriuretic peptide family (33) and is the most abundant ANP receptor in the tissues and cells that receive a large fraction of the cardiac output, including vascular endothelial (21) and smooth muscle cells (28). Thus the expected function of NPR-C is to remove natriuretic peptides from the blood circulation by binding and internalization (17). On the other hand, several lines of evidence suggest that NPR-C may have a signaling function of its own. Ring-deleted analogs of ANP that interact only with NPR-C inhibited the adenylate cyclase-adenosine 3′,5′-cyclic monophosphate system via G proteins (1, 2). NPR-C-mediated inhibition of aortic smooth muscle cell proliferation and thymidine kinase activity was also reported (4). Moreover, NPR-C activation stimulated phospholipase C activity (25).

The lung has the highest tissue concentration of specific binding sites for ANP in the rat (23), and the majority of pulmonary vascular ANP binding sites in isolated perfused rat lungs are NPR-C (19). Recent studies show that hypoxia not only increases cardiac ANP synthesis and release (31) but also lowers the metabolic clearance rate of ANP (18). Moreover, selective downregulation of NPR-C gene expression in the lung of rats adapted to hypoxia is also reported (15). These results suggest that the alteration in pulmonary NPR-C gene expression may play a role in regulating circulating ANP levels. Recently, Yanaka and colleagues (34, 35) reported the structure of the 5′-flanking regulatory region of the mouse and human genes encoding NPR-C to investigate the transcriptional regulation in detail.

Because fundamental to understanding the roles of NPR-C in the respiratory system is a determination of its precise localization in the lung, a polyclonal antibody highly specific to NPR-C has been produced in the present study. This antibody enabled us to examine in detail the localization of NPR-C in the rat lung and trachea by an immunohistochemical technique. Expression of NPR-C was seen in the epithelial cell layers of the trachea and bronchiole, the myocardium, and the mesenchymal cells surrounding the cartilage. Interestingly, this result suggested an association of NPR-C expression with chondrogenic differentiation in vivo. We investigated NPR-C expression during chondrogenic differentiation of the clonal mouse embryonic cell line ATDC5 (3), resulting in an alteration of NPR-C expression during chondrogenesis at an early stage of endochondral bone development.

MATERIALS AND METHODS

Animals and cell cultures. Male Wistar Kyoto rats at ̴ 8–10 wk of age were purchased from Charles River Japan. The clonal mouse embryonic cell line ATDC5 and clonal osteoblastic MC3T3-E1 cells were obtained from Riken Gene
Bank (Ibaraki, Japan). The ATDC5 cells were cultured in maintenance medium (a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium and 5% fetal calf serum; Gibco BRL, Gaithersburg, MD) or in differentiation medium (maintenance medium supplemented with 10 µg/ml of bovine insulin; Wako Pure Chemical, Osaka, Japan). MC3T3-E1 cells were cultured in α-modified minimum essential medium supplemented with 10% fetal calf serum. HeLa cells were obtained from Dainippon Pharmaceutical (Osaka, Japan) and cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. These cells were maintained in a controlled atmosphere of 5% CO₂-95% air at 37°C.

Antibodies. The anti-smooth muscle actin antibody was obtained from Novocastra Laboratories (Newcastle, UK). The poly-L-lysine-based multiple antigen-peptide complex containing the sequence NH₂-RKKYRITIERRNHQEESNIGKHRELRED-SIRSHFSVA-COOH, corresponding to the 37 COOH-terminal amino acids (numbers 499–535) of the rat NPR-C sequence, was synthesized by the 9-fluorenylmethyloxycarbonyl synthesis strategy. Polyclonal antibody toward the peptide was obtained by injecting Japanese White rabbits with the peptide in Freund's complete adjuvant as reported previously (13).

Production of human NPR-C protein. The human NPR-C cDNA was obtained from the human placenta cDNA library (N. Yanaka, unpublished data). To obtain a human NPR-C cDNA fragment for expression, we performed a polymerase chain reaction with primers designed according to the sequence reported previously (24), with the human placenta cDNA as a template. Primer set 1 (the sense direction primer 1 was 5'-CCCTCGAGGCGCTGCCGCCACAGAAGATCG-3' and the antisense direction primer 2 was 5'-GGGCGGCCGCTTAAGCTACT-GAAAAATGGGATCTG-3') and primer set 2 (the sense direction primer was primer 1 and the antisense direction primer 3 was 5'-GGCGGCGCGCTATTCTTCTAGGACCTGAATTGCC-3') were employed to amplify the cDNAs encoding the entire

Fig. 1. Antigenic specificity of anti-natriuretic peptide clearance receptor (NPR-C) antibody. Cell lysates prepared from E. coli cells carrying pGSThNPRC1 (lanes 1 and 3) or pGSThNPRC2 (lanes 2 and 4) were subjected to 10% SDS-polyacrylamide gel electrophoresis. Gel was analyzed by immunoblotting with anti-NPR-C antibody (lanes 1 and 2) or with the same antibody preabsorbed with an immunogenic peptide (lanes 3 and 4). Nos. at right, molecular-mass markers. Expected size of glutathione S-transferase (GST)-human NPR-C fusion protein is ~80 kDa.

Fig. 2. Immunoblot analysis of NPR-C in rat lung, MC3T3-E1 cells, and HeLa cells. A: 40 µg of membrane fractions from rat lung were subjected to 10% SDS-polyacrylamide gel. Gel was analyzed by immunoblotting with anti-NPR-C antibody (lane 1) or with the same antibody preadsorbed with an immunogenic peptide (lane 2). B: 20 µg of membrane fractions from MC3T3-E1 cells (lane 1) and HeLa cells (lane 2) were subjected to 10% SDS-polyacrylamide gel. Immunoblot analysis was performed to know whether anti-NPR-C antibody has cross-reactivity to mouse and human NPR-C proteins. Nos. at right, molecular mass markers.
human NPR-C and the truncated NPR-C that lacked 60 COOH-terminal amino acid residues, respectively. Denaturing, annealing, and polymerase reaction were done 30 times at 94°C for 1 min, at 50°C for 2 min, and at 72°C for 3 min, respectively. The amplified 1,490- and 1,310-base pair (bp) DNA fragments were digested with XhoI plus NotI and were then inserted into the corresponding sites of pBluescriptII (CLONTECH, Palo Alto, CA), producing pBLhNPRC1 and pBLhNPRC2, respectively. The nucleotide sequence was confirmed by the dideoxy chain termination method. The XhoI-NotI fragments from pBLhNPRC1 and pBLhNPRC2 were subcloned into the glutathione S-transferase (GST)-fusion protein expression vector pGEX-5X-3 (Pharmacia, Uppsala, Sweden) that was digested with SalI plus NotI, resulting in pGSThNPRC1 and pGSThNPRC2, respectively. The GST-fusion proteins were produced in Escherichia coli J M109 cells carrying these plasmids by isopropyl-β-D-thiogalactopyranoside induction as previously described (27).

Immunoblot analysis. Membrane fractions were prepared from the lungs of rats. The lungs were dissected, washed in ice-cold phosphate-buffered saline (PBS), pH 7.2, and frozen in liquid nitrogen. They were homogenized with a homog-

enizer in ice-cold homogenization buffer [(in mM) 250 sucrose, 1 EDTA, 3 MgCl₂, and 50 tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, containing the protease inhibitor 0.1 phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was centrifuged at 1,000 g for 20 min at 4°C to remove nuclei and cell debris. The supernatant was further centrifuged at 100,000 g for 1 h at 4°C. Membrane fractions from ATDC5, MC3T3-E1, and HeLa cells were also prepared. After aspiration of the culture medium, these cells were washed three times with PBS, pH 7.2, at room temperature, harvested in ice-cold homogenization buffer [(in mM) 2 dithiothreitol, 5 EDTA, 1 iodoacetic acid, and 20 Tris-HCl, pH 7.5, containing the protease inhibitor 0.2 PMSF], and then sonicated for 1 min. The homogenate was centrifuged at 1,000 g for 5 min at 4°C to remove cell debris. The supernatant was further centrifuged at 100,000 g for 1 h at 4°C.

These pellets (which contain the cell membranes) were suspended in the ice-cold 50 mM Tris-HCl, pH 7.5. Protein concentrations were determined with a protein assay kit (Bio-Rad, Melville, NY) with bovine serum albumin as the standard. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, the membrane preparations were denatured by heating at 99°C for 5 min in sample buffer [2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, 60 mM Tris-HCl, pH 6.8, and 10% (vol/vol) glycerol]. The proteins were separated by electrophoresis on 10% gels and were transferred onto Immobilon-P (Millipore, Bedford, MA). The membranes were blocked for 12 h at 4°C in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), followed by incubation with anti-NPR-C antibody (diluted 1:2,000 in PBS containing 0.1% Tween 20). Then they were washed and incubated with horseradish peroxidase-conjugated secondary antibodies, and the bound antibody was detected with enhanced chemiluminescence reagents from Amersham (Little Chalfont, UK). The specificity of the immunoblot was determined by incubation with the anti-NPR-C antibody that was previously incubated overnight at 4°C in the presence or absence of a purified immunogenic peptide.

Immunohistochemistry. Freshly dissected tissues were fixed overnight with 10% Formalin in 0.1 M phosphate buffer, pH 7.2. The tissues were dehydrated in graded ethanol solutions and embedded in paraffin. Four-micrometer sections were cut and mounted on Superfrost Plus slides (Fisher Scientific, Springfield, MA). The paraffin was removed with xylene, and the tissues were rehydrated through graded ethanol to water. Endogenous peroxidase was blocked by 0.3% hydrogen peroxide in methanol for 20 min at room temperature. Non-specific antibody staining was blocked by incubation with 1.5% normal goat serum in PBS for 1 h at room temperature in a humidified atmosphere. The sections were incubated with the purified anti-NPR-C antibody or preimmune serum (diluted 1:1,000 in PBS containing 1.5% normal goat serum) overnight at 4°C. After three washes of 10 min each in PBS containing 0.1% Tween 20, the sections were incubated with biotinylated goat-anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA) for 30 min at room temperature and washed three times, followed by incubation for 30 min at room temperature with the avidin-biotin peroxidase complex (Vector Laboratories). After three washes, visualization was carried out by incubating the sections with a solution of 50 mM Tris-HCl, pH 7.5, 0.1%, 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical), and 0.03% hydrogen peroxide. The sections were stained with hematoxylin before they were mounted.

Alcian blue staining. ATDC5 cells were maintained in six-well plates in differentiation medium. They were stained with 0.1% Alcian blue 8GS (Fluka, Buchs, Switzerland) at each time point by the method described by Shukunami et al. (30).

RESULTS

Production of human NPR-C protein and immunoblot analysis. A polyclonal antibody toward the synthetic peptide corresponding to the cytoplasmic domain
(amino acid residues 499–535) of the rat NPR-C protein has been obtained. The antigenic specificity was investigated with E. coli cells that express the entire or the truncated human NPR-C protein because the rat and human NPR-C proteins have their high sequence similarity in the cytoplasmic tail. E. coli cells carrying pGSThNPRC1 and pGSThNPRC2, which encode GST proteins fused with the entire human NPR-C and the truncated NPR-C lacking the 60 COOH-terminal amino acid residues, respectively, were obtained as described in MATERIALS AND METHODS. Cell lysates of the E. coli cells carrying these plasmids were electrophoretically separated, blotted to membranes, and incubated with the anti-NPR-C antibody or the antibody previously reacted with immunogenic peptide (Fig. 1). Although no signal was observed in the lysates of cells expressing the truncated NPR-C, a band of ~80 kDa was detected in the case of the GST-NPR-C fusion protein including the COOH-terminal moiety and was diminished after competition with a preadsorbed anti-NPR-C antibody. This result showed that the antibody specifically bound to the cytoplasmic tail of the NPR-C protein. Immuno- blot analysis of membrane fractions from the rat lung also gave the same result, a specific staining of an ~63-kDa band (Fig. 2A). This antibody was, therefore, considered to be specific to NPR-C and not to react with any other proteins in rat lung. The antibody also reacted with the NPR-C of mice and humans (Fig. 2B), demonstrating that it is a useful tool for immunohistochemical analysis.

Immunohistochemical analysis. The localization of NPR-C in the lung and tracheal airway was demonstrated by an immunohistochemical technique. Positive immunoreaction was observed predominantly in the epithelial cell layers of the trachea (Fig. 3A). In the hyaline cartilage, the chondrocytes were weakly stained, but the surrounding mesenchymal cells, especially those in the perichondrium, were strongly immunoreactive (Fig. 3A). The epithelial cell layers of bronchiol e also showed strong immunoreactivity (Fig. 3C). The pulmonary vein sometimes has the myocardium surrounding its adventitia. This myocardium was stained (Fig. 3E). Figure 3G shows the immunostaining pattern for the monoclonal anti-smooth muscle actin antibody. Positive staining was observed in the smooth muscle cells surrounding the bronchiole and artery, revealing localization of the smooth muscle cells in this section. Compared with Fig. 3E, a positive immunoreaction with the anti-NPR-C antibody was observed in the myocardium surrounding the adventitia of the pulmonary vein but not in the smooth muscle cells. No specific staining was observed when the sections were incubated with preimmune serum (Fig. 3, B, D, and F).

NPR-C expression during chondrogenic differentiation of ATDC5 cells. ATDC5 cells retain the properties of chondroprogenitor cells (3). Proliferation of ATDC5 cells halts at the confluence stage like other cell lines but in the presence of insulin restarts again, and the cells are induced to chondrogenic differentiation through a cellular condensation process, resulting in the formation of cartilage nodulelike cell aggregates (3). ATDC5 cells were inoculated into six-well plates on day 0 and cultured in differentiation medium to induce chondrogenic differentiation in vitro. Membrane fractions of ATDC5 cells were prepared on days 2, 5, 16, 21, and 36, then the cells were stained with Alcian blue at the same time point. Because cartilage nodulelike cell aggregates are stained with Alcian blue (3), we can visually recognize the progress of chondrogenic differentiation (Fig. 4). As we expected, the staining area was not found on day 2 (subconfluent stage) or on day 5 (confluent stage). Positive staining appeared on day 15, and, finally, almost all the areas were stained on day 36, indicating that ATDC5 cells were induced to chondrogenic differentiation as already reported (30). Immunoblot analysis shows the alteration of NPR-C expression during chondrogenic differentiation (Fig. 5). A band of ~63 kDa was specifically detected in the
membrane fractions on days 2 and 5. The band, however, significantly became faint on day 15 when Alcian blue staining area appeared. This result suggests that the undifferentiated ATDC5 cells express NPR-C much more than the differentiated ATDC5 cells. The changes in NPR-C expression during chondrogenic differentiation in vitro were as expected as demonstrated by immunohistochemical analysis showing the immunostaining pattern in the cartilage.

**DISCUSSION**

In this study, the precise localization of NPR-C in the rat lung and trachea was visually revealed with the anti-NPR-C antibody. First of all, expression of NPR-C was seen in the cells that are related to the circulatory system, such as the myocardium surrounding the pulmonary vein. Because the myocardium in the rat lung is considered to be from the left atrium, this finding strongly supported the assertion that NPR-C mRNA in the rat heart is concentrated principally in the atria and suggested that NPR-C mRNA transcripts in rat atria are probably translated into functional receptors and that natriuretic peptide release may be regulated in the atria. Although the lung is the target organ where plasma ANP is metabolized, no positive signal was seen in the pulmonary vascular endothelium and the alveoli where a high degree of functional activity has been demonstrated. There still remains the possibility of NPR-C expression in these cells at an undetectable level in immunohistochemical analysis. Because of its expression over a wide surface area in the lung, NPR-C may play a role in the clearance and metabolism of the natriuretic peptides. In addition, immunohistochemical analysis demonstrated that the cells seem to be unrelated to the circulatory system, such as the epithelial cell layers of the trachea and bronchioles. The lung is a site of synthesis and release of ANP. The finding of NPR-C expression outside the pulmonary circulation suggests that the natriuretic peptides may be involved in regulating other functions of the respiratory system.

Interestingly, immunostaining was observed both in chondrocytes and in mesenchymal cells in the cartilage. The immunostaining pattern of the hyaline cartilage showed high NPR-C expression in mesenchymal cells, especially those in the perichondrium, and low expression in matured chondrocytes. The hyaline cartilage grows as a result of the secretion of characteristic cartilage proteoglycans from chondrocytes at first, but the main way of cartilage growth changes to what is called the appositional growth with development. That is, the mesenchymal cells in the perichondrium differentiate to the chondrocytes; thus the hyaline cartilage grows by adding chondrocytes from the outside. We hypothesized that NPR-C expression is associated with chondrogenic differentiation. The mouse embryonal carcinoma-derived donal cell line ATDC5 has been employed to prove this hypothesis. ATDC5 cells exhibit a fibroblastic, mesenchymal morphology, but the chondrogenic differentiation is induced by insulin stimulation. Immunoblot analysis indicated that NPR-C expression was intensively seen in the undifferentiated ATDC5 cells but decreased in the differentiated cells. This result has clarified the hypothesis. Hagiwara et al. revealed the absence of NPR-C in the chondrocytes in rat xiphoid cartilage and the marked increase in its expression during in vitro culture, indicating that the dedifferentiated chondrocytes come to express NPR-C. Here we demonstrated downregulation of NPR-C expression during chondrogenic differentiation; that is, undifferentiated chondrocytes as well as dedifferentiated chondrocytes express NPR-C. Thus NPR-C is considered to be a useful marker representing the chondrocytes in an undifferentiated or dedifferentiated stage. Although the role of NPR-C in chondrogenic differentiation remains unclear, the natriuretic peptide system may be involved in endochondral bone formation as well as in osteoblastic differentiation.

We have produced the anti-NPR-C antibody and examined the localization of NPR-C in the rat lung and trachea using the antibody. Immunoreactivity with human NPR-C is a quite important property for the pathological analysis of the NPR-C protein. Further investigations may lead to a better understanding of the biological functions or the signal transduction mechanism of NPR-C that was originally thought to be just a clearance receptor for natriuretic peptides.

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