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Human androgen-induced growth factor in prostate and breast cancer cells: its molecular cloning and growth properties

Akira Tanaka[a], Kaoru Miyamoto[b], Hisayuki Matsuo[b], Keishi Matsumoto[c], Hiroki Yoshida[a,*]

[a]*Department of Pathology, Faculty of Medicine, Kagoshima University, Kagoshima 890, Japan*
[b]*National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan*
[c]*Osaka Medical Center for Maternal and Child Health, Izumi, Osaka 590-02, Japan*

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Abstract Androgen-induced growth factor (AIGF) has hormone-regulated properties in the mouse Shionogi carcinoma cell line. To investigate whether or not it is involved in growth of human hormone-responsive cancers, we isolated the human AIGF gene from a placental genomic library. Genomic analyses suggested that the AIGF gene was about 6.5 kilobases in length containing five exons. The deduced amino acid sequence of human AIGF was completely identical with that of the mouse. RT-PCR analyses showed that prostate and breast cancer cell lines, LNCaP, PC-3, and MCF-7, slightly expressed the AIGF gene. Recombinant AIGF enhanced the growth of the human prostate cancer LNCaP cells, and it also markedly stimulated the growth of fibroblasts. These in vitro findings suggest that AIGF might be a possible autocrine or paracrine factor in hormone-responsive cancers.

Key words: Fibroblast growth factor family; Molecular cloning; Prostate cancer; Breast cancer

1. Introduction

The fibroblast growth factor (FGF) family is involved in various biological processes such as animal development, neurogenesis, and angiogenesis (reviewed in [1]). Nine members have been identified to date. Acidic and basic FGFs are prototypal and they play various important roles [2]. Int-2/FGF-3, Hst-1/FGF-4, FGF-5, and FGF-6 were originally isolated as oncogene products [3,4,5,6], and are mainly expressed at various developmental stages in the normal embryo [7,8,9,10]. Androgen-induced growth factor (AIGF)/FGF-8 has been isolated from the conditioned medium of the androgen-dependent mouse mammary Shionogi carcinoma cell line (SC-3) stimulated with testosterone [11]. AIGF is secreted by SC-3 cells in response to androgenic stimuli, and then it stimulates the growth of the cells in an autocrine manner [11]. Since AIGF has hormone-inducible and oncogenic features in SC-3 cells, AIGF is suspected to play important roles on the growth of human hormone-responsive breast and prostate cancers. In addition, FGFs or FGF receptors (FGFRs) are often overexpressed in various types of carcinomas and seem to contribute to the progression of these tumors [12,13,14]. Especially basic FGF is well-known to be an important factor for benign and malignant growth of prostate cells [15,16,17], and keratinocyte growth factor (KGF)/FGF-7 is recently suggested to be involved in mesenchymo-epithelial paracrine interaction in the normal prostate and prostate cancers [1,18]. These findings have led us to investigate whether or not AIGF is involved in the hormone-responsive growth of prostate and breast cancers. In this study, we first present molecular cloning of the human AIGF gene. And then, we show possible autocrine or paracrine functions of AIGF in human prostate and breast cancer cells.

2. Materials and methods

2.1. Cell lines

LNCaP, PC-3, ZR75-1 and MCF-7 cells were purchased from Dainippon Pharmacy Corp. (Osaka, Japan). NIH 3T3 cells were gifted from National Cardiovascular Center Research Institute. LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). PC-3 cells were maintained in Ham's F12-K medium containing 10% FBS. MCF-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) medium supplemented with 10% FBS and 10 nM 17β-estradiol. ZR75-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS. NIH 3T3 cells were maintained in DMEM medium containing 10% FBS. SC-3 cells were cloned and maintained as reported [11]. During studies of hormone stimulation, intrinsic steroids were stripped from FBS using dextran-coated charcoal.

2.2. RNAs

Total cellular RNAs were extracted from subconfluent testosterone-stimulated or non-stimulated LNCaP and PC-3 cells using acid guanidium isothiocyanate–phenol–chloroform [19]. Total cellular RNAs were also extracted from subconfluent MCF-7, ZR75-1 and testosterone-stimulated SC-3 cells.

2.3. Southern blotting

DNAs were electrophoresed in agarose gels and transferred onto nylon membranes. Hybridization was performed in 5 × SSPE (1 × SSPE = 0.15 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA) containing 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone) and 0.5% SDS at 65°C for 18 h. Blots were subsequently washed with 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS at 50°C for 30 min and 0.1 × SSC containing 0.5% SDS at 50°C for 30 min.

2.4. Isolation of human AIGF genomic clones

A human placental genomic phage library, purchased from Stratagene (La Jolla, CA), was screened. In total, 5×10^5 clones were screened using a ^{32}P-labeled whole mouse AIGF cDNA probe. Isolated DNAs were subcloned into the Blue Script II vector (Stratagene) for further analysis.

2.5. Nucleotide sequencing

Restriction enzyme mapping and Southern blotting showed that most of the positive clones contained similar fragments hybridized with the mouse AIGF probe. The four hybridized fragments shown in Fig. 1A (1.0 kb *Not*I-*Pst*I, 0.7 kb *Pst*I-*Pst*I, 0.8 kb *Bam*HI-*Kpn*I, and 1.5 kb *Xho*I-*Sac*I fragments) were subcloned into the Blue Script II vector and sequenced by dideoxynucleotide chain-termination [20] using a cycle

*Corresponding author. Fax: (81) (992) 64-6348.

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sequencing kit and a DNA sequencer (Applied Biosystems Division, Perkin Elmer Cetus, Norwalk, CT). The sequence was determined on both strands.

2.6. RT-PCR

Two primer sequences were used to amplify human and mouse AIGF: the 5′-primer was 5′-TTTACACAGCATGTGAGGGAG-3′ (nucleotide sequences from 264 to 284 in Fig. 1B) and the 3′-primer was 5′-GTAGTTGAGGAACTCGAAGCG-3′ (nucleotide sequences from 758 to 738). Both sequences are common to human and mouse AIGFs. Glyceraldehyde 3-phosphate dehydrogenase (G-3-PDH) control primers which are common to human and mouse were purchased from Clontech (Palo Alto, CA). The cDNA was synthesized from 500 ng of total RNA using the 3′-primer and the RT-PCR kit (GeneAmp RNA PCR Kit; Perkin Elmer Cetus). Samples were cycled for PCR 35 times at 95°C for 1 min, at 55°C for 1 min, and 72°C for 1 min with total volume of 100 μl. Ten μl of PCR-amplified samples were electrophoresed in 2% agarose gels, and stained with ethidium bromide. These fragments were further analyzed by Southern blotting using the whole mouse AIGF cDNA probe. The Intensity of the hybridized AIGF band of each lane was measured by a laser densitometer (Pharmacia LKB, Uppsala, Sweden). Some of the PCR-amplified fragments were purified and ligated into the pGEM-T vector (Promega, Madison, WI). The nucleotides of the amplified fragments were sequenced as described above.

2.7. Preparation of recombinant AIGF

Since amino acid sequences of human and mouse AIGF are completely identical, recombinant AIGF was prepared from an isolated stable cell line transfected with the mouse AIGF cDNA. Namely, the mouse AIGF cDNA was subcloned into the eukaryotic expression vector, pSD(X)-dhfr, containing the mouse dihydrofolate reductase gene as a selective marker. The resulting expression plasmid, pSD(X)-dhfr-AIGF, was transfected into CHO(dhfr−) cells by the calcium phosphate precipitation method. Recombinant AIGF was purified from the supernatant of the isolated clonal CHO cell line as reported [11].

2.8. Cell proliferation assay

LNCaP and PC-3 cells (4×10^4 cells/dish) were plated onto 35 mm-dishes containing 1.5 ml of Ham's F12: Eagle's minimum essential medium (MEM) (1:1; v/v) supplemented 2% FBS treated with dextran-coated charcoal. On the following day (day 0), the medium was changed to 1.5 ml of the medium with or without 10 ng/ml basic FGF (Pepro Tech Inc., Rocky Hill, NJ), 10 ng/ml recombinant AIGF, or 10 nM testosterone. The medium was changed every other day. Three or four dishes were used for each samples. Cells were counted on day 5. On the other hand, NIH 3T3 cells (5×10^3 cells/well) were plated onto a 24-well plate containing 1 ml of RPMI 1640 supplemented with 2% FBS. After 5 h plating, the medium was changed to the medium with or without test compounds. The medium was changed every other day. Three wells were used for each samples. Cells were counted on day 6.

3. Results

3.1. Isolation and characterization of the human AIGF gene

Genomic analyses showed that the human AIGF gene was 6.5 kb in length and that it contained five exons (Fig. 1A). Highly conserved sequences between mouse and human AIGF allowed us to tentatively determine the human AIGF cDNA sequence (Fig. 1B). Later, sequencing of the amplified RT-PCR fragments verified the nucleotide sequence from 264 to 758. The deduced amino acid sequence of human AIGF was completely identical with that of the mouse.

3.2. RT-PCR analysis for human AIGF expression in prostate and breast cancer cell lines

The expression of AIGF in human prostate or breast cancer cell lines was examined by means of RT-PCR. In the androgen-responsive and unresponsive prostate cancer cell lines, LNCaP and PC-3, respectively, low levels of amplified AIGF fragments were detected in the presence or absence of testosterone (Fig. 2A). Measured by a laser densitometer, the levels of AIGF expression of both cell lines under testosterone-stimulated or non-stimulated conditions were almost similar. Compared with that of SC-3 cells, the densitometric levels of these lanes were about 40%. However, no growth-promoting activity was found in their conditioned media when assayed by SC-3 cells (data not shown). The amplified AIGF fragment was also detected in the estrogen-responsive breast cancer cell line, MCF-7, but not ZR75–1, which is also an estrogen-responsive breast cancer cell line (Fig. 2B).

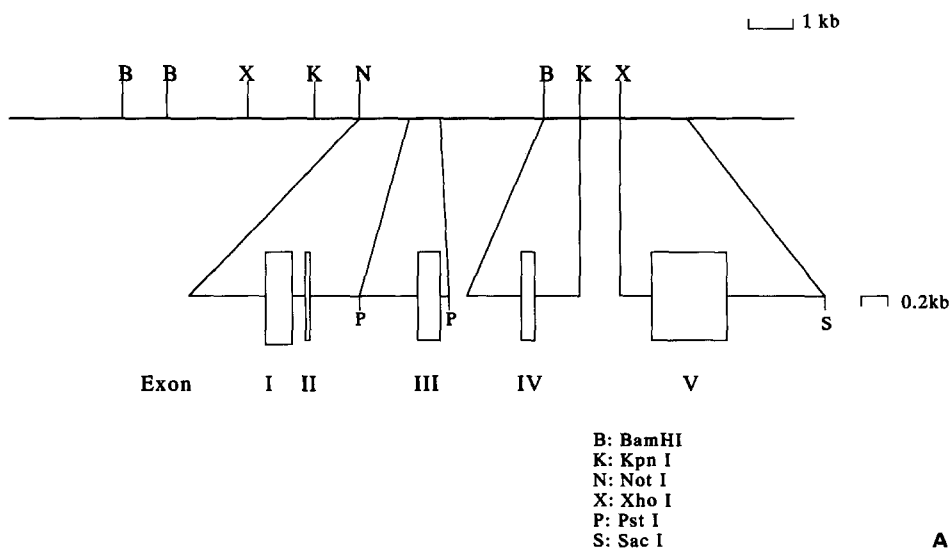

Fig. 1. (A) Restriction enzyme map of the human AIGF locus. Subclones used for nucleotide sequencing are shown below. Five identified exons are boxed. Abbreviations for the restriction enzymes sites are: B, *Bam*HI; K, *Kpn*I; N, *Not*I; X, *Xho*I; P, *Pst*I; S, *Sac*I.

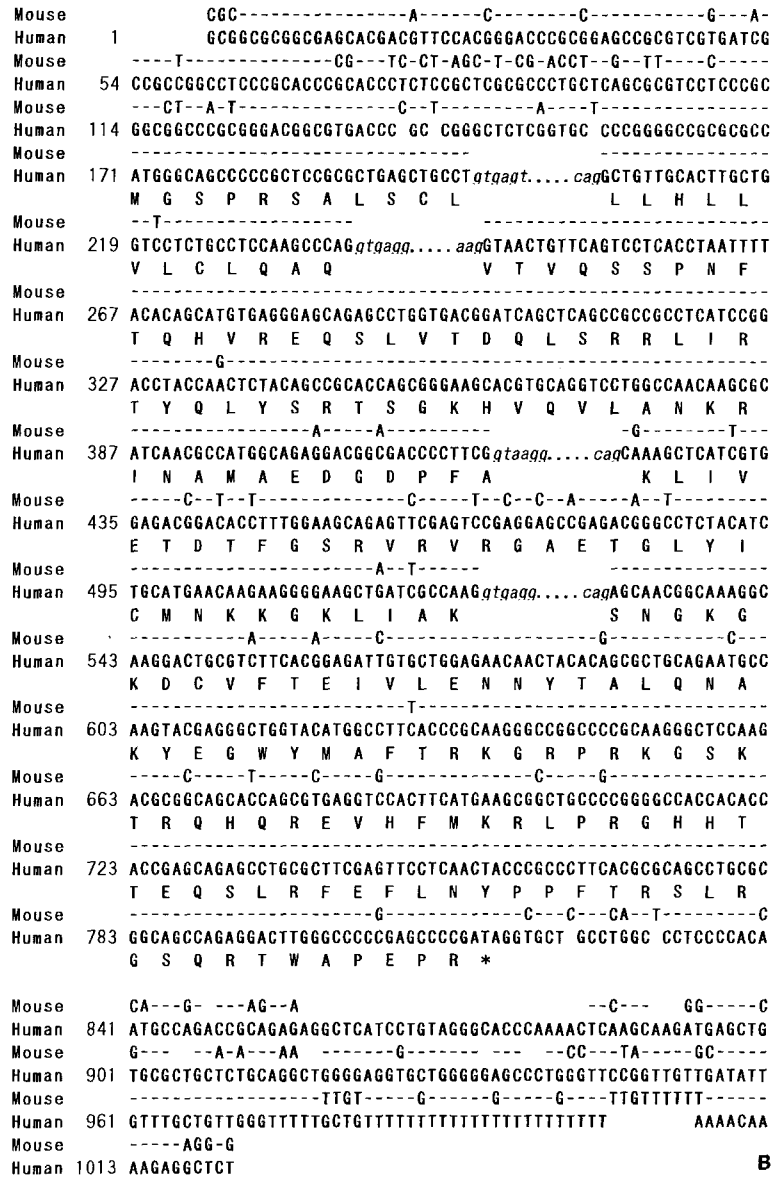

Fig. 1. (B) Nucleotide and amino acid sequences of human AIGF compared with the mouse. Human nucleotide sequences are numbered according to the numbers of mouse AIGF cDNA [11]. The exon–intron boundaries are shown in italics. Identical nucleotide sequences are shown in dashes. Gaps were introduced to maximize homology.

3.3. *Effects of recombinant AIGF on growth of cultured cell lines*

Preliminary experiments using the purified recombinant AIGF showed that it markedly stimulated the growth of SC-3 cells in a dose-dependent manner and its ED_{50} value was about 10 ng/ml (data not shown). When stimulated by 10 ng/ml recombinant AIGF, 10 ng/ml basic FGF, or 10 nM testosterone, the growth of LNCaP cells were enhanced up to the levels of 150%, 230% or 300% , respectively, compared with the control (100%) in cell number. PC-3 cells were not stimulated by these compounds. The growth of NIH 3T3 cells was markedly stimulated in the presence of 10 ng/ml recombinant AIGF or 10 ng/ml basic FGF up to the level of 340% or 200%, respectively, compared with the control (Fig. 3).

4. Discussion

The nucleotide sequences of AIGF were highly conserved between mouse and human. There were only 30 silent nucleotide changes in the coding region compared with mouse AIGF. The deduced amino acid sequence of human AIGF was completely identical with that of the mouse. The high degree of amino acid homology implied that AIGF had a highly conserved function. Recently, Ohuchi et al. have suggested from their in situ hybridization studies that AIGF plays important biological roles on brain development or limb and facial morphogenesis [21]. More studies such as homologous recombination experiments will elucidate distinct biological roles of AIGF.

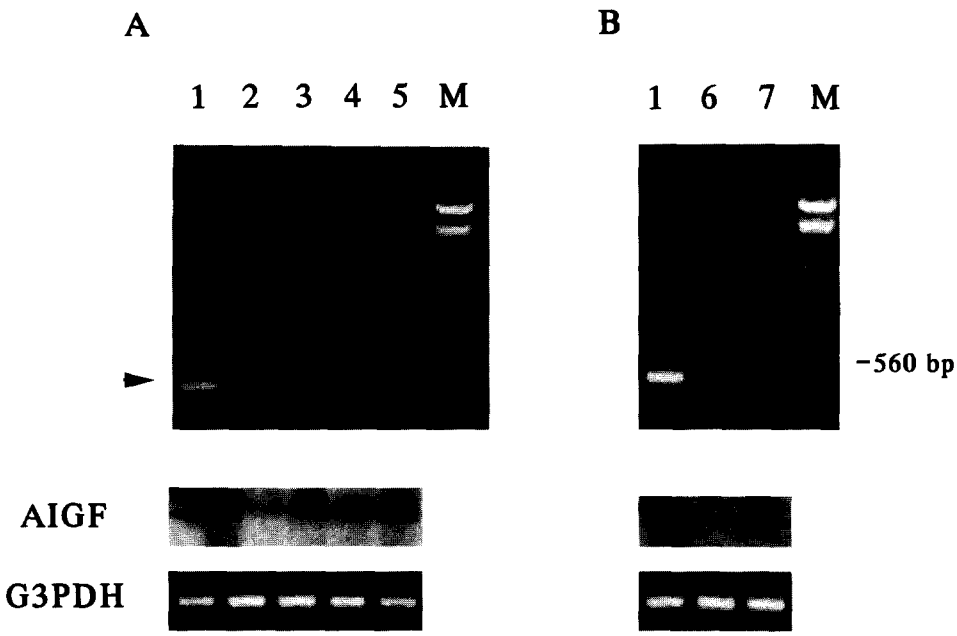

Fig. 2. RT-PCR analysis of AIGF expression in various human prostate and breast cancer cell lines. (A) RT-PCR analysis of AIGF expression in the human prostate cancer cell lines, PC-3 and LNCaP. Total RNAs obtained from the PC-3 or LNCaP cells stimulated or not with testosterone were reverse-transcribed and the cDNAs were amplified 35 cycles of PCR by using AIGF or G-3-PDH primers as described in section 2. RT-PCR products were electrophoresed in agarose gels, stained with ethidium bromide and analyzed by Southern blotting using the whole mouse AIGF cDNA probe. The arrow indicates the position of AIGF. SC-3 cells were used for the positive control. Lane 1 = SC-3 cells; lane 2 = testosterone-stimulated PC-3 cells; lane 3 = non-stimulated PC-3 cells; lane 4 = testosterone-stimulated LNCaP cells; lane 5 = non-stimulated LNCaP cells; M = molecular weight marker. (B) RT-PCR analysis of AIGF expression in human breast cancer cell lines, MCF-7 and ZR75-1. AIGF expression was also examined by RT-PCR in both breast cancer cells. Lane 1 = SC-3 cells; lane 6 = ZR75-1 cells; lane 7 = MCF-7 cells; M = molecular weight marker.

In general, genes of the FGF family contain three exons with similar exon-intron boundaries [3,4,6,22]. Exons IV and V of the AIGF gene are well correlated with the second and third exons of other FGF genes. On the other hand, the equivalent of the first exon of other FGF genes is divided into three small exons (exons I, II, and III in Fig. 1A) in the AIGF gene. Previously, using the SC-3 mouse mammary cancer cell line, we reported an isoform of mouse AIGF which was alternatively spliced at the 5′-upstream portion of the exon III [11]. In this study, the isoform was not precisely determined even after full sequencing of the second intron (data not shown).

We previously demonstrated that AIGF mediates the androgen-dependent growth of the mouse mammary Shionogi carcinoma SC-3 cells [11]. The findings obtained from these cells strongly suggested that hormone-induced growth factors are also involved in human prostate and breast cancers. Many investigators have reported that various growth factors are involved in growth of these cancers (reviewed in [23,24]). However, no distinct hormone-induced growth factors have yet been identified in human prostate and breast cancers as far as we know. Although KGF/FGF-7 is suggested to be a paracrine growth factor which is secreted from stromal cells in response to androgenic stimuli in a rat prostate cancer model [18], its involvement of the human cancers have not been elucidated yet. In this study, we presented that AIGF was slightly expressed in the prostate cancer cell lines, LNCaP and PC-3, under both testosterone-stimulated and non-stimulated conditions. The cell growth experiments showed that LNCaP cells actually increased in cell number, responded to exogenous AIGF. These results showed possibilities that AIGF could make an autocrine loop in prostate cancers. However, unlike the mouse mammary

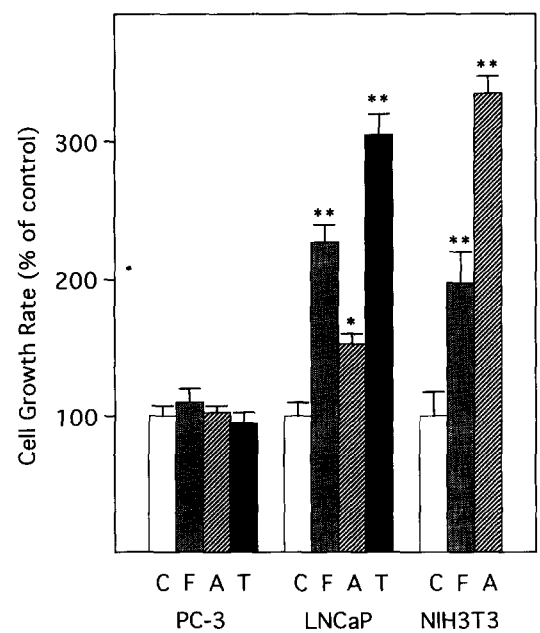

Fig. 3. Effects of recombinant AIGF (A; 10 ng/ml), basic FGF (F; 10 ng/ml), or testosterone (T; 10 nM) on growth of LNCaP, PC-3, and NIH 3T3 cells. LNCaP, and PC-3, and NIH 3T3 cells were plated, cultured, and examined for growth as described in section 2. Results were expressed as percent of each control (C). Actual mean numbers of the controls were: LNCaP, 5.0×10^4; PC-3, 24.8×10^4: NIH 3T3, 14.6×10^5. Values = means of 3–4 determinations; bars = S.E.M.; $^*P < 0.01$, $^{**}P < 0.001$, when compared to each control. The other trial gave similar result.

Shionogi carcinoma cells, hormone-regulated properties of AIGF were not clear in the human prostate cancer cells. More studies will be expected to elucidate the actual properties of AIGF. The cell growth experiments also showed that AIGF was a strong mitogen for fibroblasts. Ohuchi et al. suggested in their developmental study that AIGF should be an epithelial factor that mediates epithelio-mesenchymal interactions [21]. Although specific functions of AIGF have not yet been elucidated, it is possible that AIGF may act as a paracrine growth factor on cancer growth.

In summary, we presented here, using several human cancer cell lines, that AIGF might be a possible autocrine or paracrine factor in hormone-responsive cancers. However, hormone-regulated properties of AIGF was not demonstrated in this study. More extensive studies using human surgical materials will provide more informations about actual properties of AIGF in human hormone-responsive cancers.

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