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Minireview

Molecular Mechanism of Androgen-dependent Growth in Transformed Cells. Pathway from Basic Science to Clinical Application

Masafumi Koga,[1] Soji Kasayama,[1] Keishi Matsumoto[2] and Bunzo Sato[3]*

[1]Department of Medicine III, Osaka University Medical School, 2-2 Yamadaoka Suita-shi 565, [2]Osaka Medical Center and Research Institute for Maternal and Child Health, 840 Murodou-cho, Izumi-shi 590-02 and [3]The Third Department of Internal Medicine, Nissei Hospital, 6-3-8 Itachibori Nishi-ku, Osaka 550, Japan

Shionogi carcinoma 115 (SC 115) has been extensively used to analyze the mechanism of androgen-dependent cancer growth. This tumor exhibits marked androgen-dependent growth *in vivo* and one cell line whose growth is markedly stimulated by androgen in serum-free culture condition is isolated from SC 115 tumor. This androgen-dependent growth is mediated through an induction of heparin binding growth factor termed as androgen-induced growth factor (AIGF). In addition, fibroblast growth factor receptor 1 (FGFR 1) is identified as a receptor for AIGF. The expression of FGFR 1 mRNA is up-regulated by androgen in SC 115 cells, indicating that this androgen-inducible autocrine loop is potentiated at two sites by androgen. An androgen-independent cell line is also established from this androgen-dependent SC 115 tumor. The growth of these androgen-independent cells is stimulated by AIGF, indicating that AIGF acts not only as an autocrine growth factor to androgen-dependent cells but also as a paracrine growth factor to androgen-independent cells. In addition, transfection of AIGF expression vector into androgen-dependent cells results in a facilitation of conversion from androgen-dependent to -independent phenotype. Thus, AIGF might play a role from tumor progression. These results indicate that a blockade of AIGF activity is an important therapeutic target. Actually, some compounds such as heparin and suramin are found to inhibit this androgen-induced autocrine loop. These basic observations will be discussed in relation to their possible clinical application.

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A BRIEF HISTORY OF SHIONOGI CARCINOMA 115

One breast cancer spontaneously developed in DS mice was identified at the animal laboratory of the Shionogi Pharmaceutical Company. After serial transplantation (115 generations), one subline was found to grow only in male, but not female, mice [1]. Thus, this subline, termed as Shionogi carcinoma 115 (SC 115), was categorized as an androgen-dependent cancer. This was confirmed by hormonal manipulation of host mice such as castration and hypophysectomy with or without androgen replacement [2]. Furthermore, SC 115 cells were demonstrated to contain a significant concentration of androgen receptor but quite a low level of 5α-reductase, indicating that the direct association of testosterone with androgen receptor results in the growth enhancement of SC 115 cells. In addition, a removal of androgen stimuli results in complete remission of SC 115 tumor when androgen ablation was initiated at the stage where the size of SC 115 tumor was quite small, whereas only a partial and no response were observed when androgen ablation was

*Correspondence to B. Sato.
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initiated at the stage of the medium and large size of tumor, respectively, indicating that these responses to hormonal manipulations are similar to those of human hormone-dependent cancer such as breast and prostate cancer and SC 115 tumor is quite suitable for analyzing an androgen-dependent tumor growth as a model system [3]. These results prompted us to analyze the molecular mechanism of androgen-dependent growth of SC 115 cells. For 10 years, we have concentrated our attention to clarify the mechanism of androgen-dependent growth of SC 115 cells. An initial attempt was made to establish the cell line from SC 115 tumor which exhibited remarkable androgen-dependent growth in serum-free culture condition. In 1986, we obtained one cell line (SC 3 cells) suitable for analysis of androgen-dependent growth at cell biology as well as molecular biology levels.

CELL BIOLOGY OF SC 3 CELLS

The growth of SC 3 cells is remarkably enhanced by androgens in HMB medium (Ham F-12:minimum essential medium; 1:1 (v/v) containing 0.1% (w/v) fatty acid-free bovine serum albumin) without any aid of serum and growth factors. The addition of 10^{-8} M testosterone in HMB medium results in more than 10-fold higher cell yield when compared with that of unstimulated cells during 7 days culture. When DNA synthesis was measured by pulse-labeling with radioactive thymidine, a 50–100-fold increase in the amount of radioactivity incorporated into DNA was elicited by androgen stimuli for 1–3 days [4]. To investigate the hormone specificity, SC 3 cells were stimulated by various hormonal steroids. Glucocorticoid could exert its growth-stimulatory ability, although relatively high concentrations (10^{-7}–10^{-6} M of dexamethasone) were required. Pharmacological doses of glucocorticoid also enhanced the growth of SC 115 tumors in mice [5]. On the other hand, the effects of estrogen on the growth of SC 115 tumor were rather complicated. Pharmacological doses of diethylstilbesterol (DES), which is known to possess affinity to the estrogen receptor, but not to the androgen receptor, were able to stimulate the growth of SC 115 tumor in mice but not the growth of SC 3 cells in the culture condition. As mentioned above, SC 115 tumor cannot grow in untreated female mice, indicating that physiological levels of estrogen are insufficient for supporting the growth of SC 115 tumor in mice. Although the explanation of these apparently contradictory results on estrogen effects at the molecular level remain unsolved, it should be stated that the administration of pharmacological doses of DES into mice is an alternative procedure for maintaining SC 115 tumors [6]. From these results, only androgen can maintain and stimulate the proliferation of SC 115 cells in mice and in cell culture at physiological concentrations.

The effects of authentic growth factors on the growth of SC 3 cells have been also investigated in serum-free culture condition. Neither insulin nor platelet-derived growth factor can exert a significant effect. Epidermal growth factor shows only a marginal effect. On the other hand, both acidic and basic fibroblast growth factor (a- and bFGF) remarkably stimulate the DNA synthesis of SC 3 cells. Interestingly, FGFs fail to show additive effects on the DNA synthesis of androgen-stimulated SC 3 cells, suggesting that both androgen and FGFs share a common signal pathway in eliciting the DNA synthesis of SC 3 cells [7]. These observations prompted us to examine the possibility that androgen exerts its growth-promoting ability through an induction of an autocrine growth factor similar to fibroblast growth factor. To address this possibility, the growth-promoting activity in conditioned medium was analyzed. We identified the activity from androgen-stimulated, but not -unstimulated, SC 3 cells, as being compatible with our speculation. Biochemical analyses revealed that this activity can be associated with and eluted at 1.1–1.3 M NaCl from a heparin-Sepharose affinity column [8]. This chromatographic behavior was slightly different from those of a- and bFGF, indicating that the growth factor involving androgen-induced SC 3 cell growth is similar to, but distinct from, authentic FGFs. These considerations might be supported by the findings that neutralizing polyclonal antibody against bFGF can block androgen-induced growth but bFGF expression fails to be demonstrated in androgen-stimulated SC 3 cells [9].

Since a role of androgen-induced growth factor (AIGF) in stimulating SC 3 cell growth became evident, we examined the effects of some compounds known as modulators of FGF actions on androgen-induced growth. Suramin which is an inhibitor of various growth factors [10] can block androgen- as well as AIGF-induced growth at much lower concentrations than those required for an inhibition of FGFs-induced growth [11]. The effects of heparin on SC 3 cell growth is also interesting. Exogenous addition of soluble heparin has been reported to potentiate FGFs action in some systems but to inhibit it in other systems [12]. In SC 3 cell culture system, heparin is observed to effectively inhibit androgen-induced growth, while FGFs-induced growth is slightly enhanced by heparin [13]. Since the cell-surfaced heparan sulfate is essentially required for FGFs-dependent growth in many cells including SC 3 cells [14, 15], our results can be interpreted as an indication that complexing with exogenously added heparin renders AIGF, but not FGFs, biologically inactive and this binding hinders AIGF from formation of active complexes with cell-surfaced heparan sulfate in a competitive manner. In addition, these results support our notion that AIGF differs from authentic FGFs in a molecular sense.

Identification of AIGF in the pathway of androgen-dependent SC 3 cell growth provides us with some clue for investigating the mechanism of progression from androgen-dependent to -independent tumor. In this

relation, androgen-independent cell line, termed as SC 4, was isolated from the same tumor mass which was used for isolation of SC 3 cells. This fact would suggest that SC 115 tumor consists of a mixture of androgen-dependent and -independent cells although its growth in mice is completely androgen-dependent as a mass. To address this interesting biological event, we analyzed the growth mechanism of SC 4 cells. SC 4 cells also secrete the growth factor in an autonomous fashion. This SC 4-derived growth factor seems to differ from SC 3-derived AIGF, judged from their chromatographic behavior on heparin-Sepharose affinity column. The proliferation rate of SC 4 cells is relatively slow, probably due to the fact that SC 4-derived growth factor is qualitatively or quantitatively insufficient for stimulating the SC 4 cell growth at the accelerated rate. Interestingly, SC 4 cells are growth-enhanced by AIGF from SC 3 cells, suggesting that AIGF can stimulate the growth of androgen-independent cells as a paracrine growth factor [16]. This might be a reason why the tumor mass consisting of a mixture of androgen-dependent and -independent cells show complete androgen-dependency. When the androgen stimuli are discontinued, the medium-sized tumor might be expected to contain the number of androgen-independent cells sufficient for producing quantitatively enough of this autonomous growth factor and regrowing as an androgen-independent tumor. These observations are schematically represented in Fig. 1.

MOLECULAR BIOLOGY OF SC 3 CELLS

Since a couple of biological and biochemical studies revealed an important role of AIGF in androgen-dependent SC 3 cell growth, we attempted the molecular cloning of AIGF cDNA. Purification of AIGF with subsequent determination of amino acid alignment of its proteolytic fragments enabled us to isolate its cDNA. AIGF consists of 215 amino acids with a putative signal sequence at N terminal region [17]. As expected from the above-mentioned biological results, AIGF contains some regions homologous to FGF family proteins with overall homology of 20–30% at amino acid level (Fig. 2). Since the homology regions are scattered on the whole molecule, AIGF can be categorized as a new growth factor but not as an alternatively spliced variant form of an already known growth factor. Using AIGF cDNA as a probe for Northern blot analysis, AIGF mRNA expression has been studied. The level of AIGF mRNA is undetectable in androgen-unstimulated SC 3 cells and becomes detectable after 3 h androgen stimulation. Further elevations are observed at 24 h of androgen stimulation. This expression pattern reveals that AIGF mRNA expression is under strict control of androgen. Glucocorticoid also induces AIGF mRNA but at much lower levels than androgen, and relatively high concentrations (10^{-7}–10^{-6} M) of dexamethasone are required to induce AIGF mRNA. Estradiol fails to induce AIGF mRNA at concentrations of less than 10^{-6} M (unpublished observation). These results are in parallel to those on steroid hormone-induced growth of SC 3 cells.

To further confirm a role of AIGF in eliciting androgen-induced growth, antisense phosphorothioate oligonucleotides encompassing the translation initiation site of AIGF mRNA were prepared to block its translation. AIGF antisense oligonucleotides effectively block androgen-induced DNA synthesis in SC 3 cells, whereas sense nucleotides are without any effect [18]. This observation indicates that AIGF is an obligatory intermediate in the androgen-induced autocrine loop.

Since AIGF cDNA has been cloned from transformed cells, its transforming activity seems to be an interesting research project. In addition, FGF family proteins with the signal sequence (int 2, hst, FGF 5 and FGF 6) have been reported to exhibit transforming activity [19]. Transfection of AIGF expression vector is found to confer the transformed phenotypes to NIH3T3 cells, based upon the data on focus formation assay, anchorage-independent growth and tumor formation in nude mice [18]. Thus, AIGF can be categorized as an oncogene.

In view of the fact that AIGF acts as an autocrine growth factor, the receptor for AIGF must be expressed on the surface of SC 3 cells. Our preliminary biochemical studies revealed that the binding of bFGF to SC 3 cells was inhibited by partially purified AIGF. These results would suggest that both AIGF and bFGF share a common receptor. Up to the present moment, four different molecules (FGF receptor 1–4) have been known as receptors for FGFs [20]. Among these molecules, SC 3 cells predominantly express FGF receptor 1 (FGFR 1). Thus, FGFR 1 is a likely candidate for AIGF receptor. To postulate this possibility, we have first attempted to isolate cDNA encoding FGFR 1 from SC 3 cells. FGFR 1 expressed in SC 3 cells has been found to contain a 12 amino acid insertion near the N-terminal region with two additional minor deviations [21]. A final conclusion that this FGFR 1 is a receptor for AIGF has been drawn from an experiment involving the transfection of this FGFR 1 expression vector into FGFR-negative rat myoblast cells (L6 cells) [18]. Transfection results in an acquisition of the ability for L6 cells to respond to AIGF. Thus, FGFR 1 is established as a receptor for AIGF. Interestingly, FGFR 1 expression in SC 3 cells is up-regulated by androgen as well as FGF stimulation, indicating that the androgen-induced autocrine loop is also potentiated at the level of FGFR 1 [21]. Our recent analysis of the FGFR 1 gene is able to clarify the region (-62 to -42 from transcription start site) essential for its promoter activity [22, 23]. One nuclear protein with a molecular mass of 100 k can be associated with this region in a sequence-specific manner. This protein cannot be identified in FGFR 1-nonexpressing cells [23]. Thus, the structure and

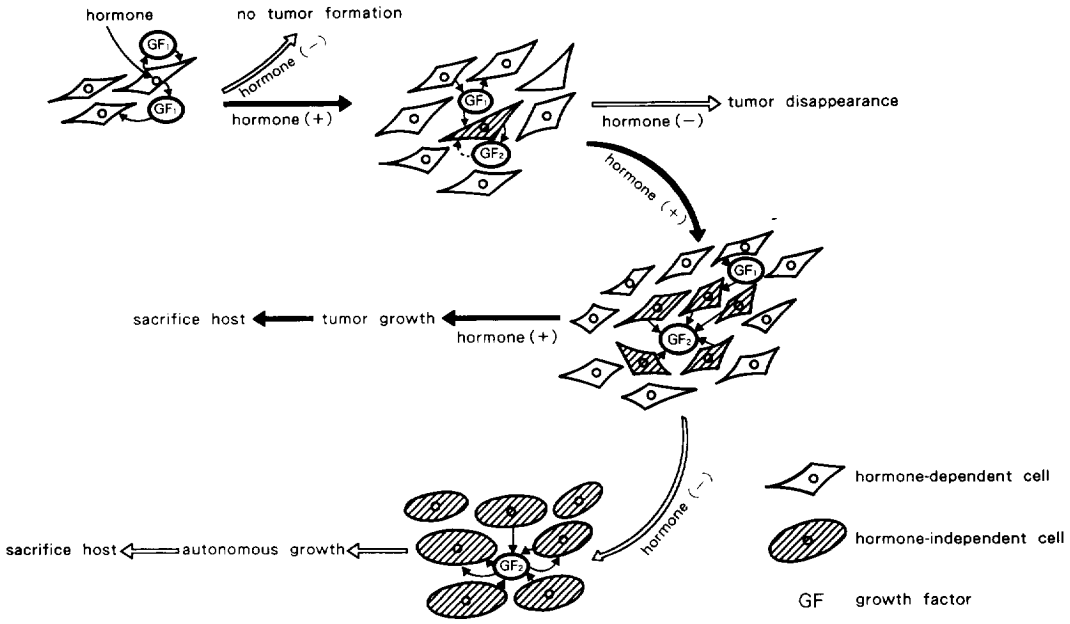

Fig. 1. Determination of androgen sensitivity of a tumor consisting of a mixture of androgen-dependent and -independent cells through autocrine or paracrine mechanisms. Transformation occurs in an androgen-dependent cell. During androgen-dependent growth mediated through an autocrine growth factor, further genetic alteration rendering the cell androgen-refractory might be induced. When androgen ablation is carried out at the early stage where the ability of androgen-independent cells to produce the growth factor qualitatively or quantitatively is insufficient for their own growth, however, the tumor might be completely regressed. When the tumor grows further and is contaminated with a relatively large number of androgen-independent cells which are able to produce enough growth factor for maintaining and proliferating these cells, androgen ablation results in only partial response and tumor regrowth. Almost all prostate cancers might be clinically identified at this stage. Please see the text for more detailed information.

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AIGF                                                    MGSPRSALSCLLLHLLVLCLQAQVTVQSSPNFTQHVREQSLVTDQL
bFGF                                                          MAAGSITTLPALPEDGGSGAFP
aFGF                                                            MAEGEITTFTALTEKFNLP
INT-2                                                  MGLIWLLLLSLLEPGWPAAGPGARLRRDAGGRGGVYEH
HST/kFGF          MSGPGTAAVALLPAVLLALLAPWAGRGGAAAPTAPNGTLEAELERRWESLVALSLARLPVAAQPKEAAVQSGAGDY
FGF-5        MSLSFLLLLFFSHLILSAWAHGEKRLAPKGQPGPAATDRNPRGSSSRQSSSSAMSSSSASSSPAASLGSQGSGLEQSSFQ
FGF-6          malqqklfitMSRGAGRLQGTLWALVFLGILVGMVVPSPAGTRANNTLLDSRGWGTLLSRSRAGLAGEIAGVNWESGY
KGF                                          MHKWILTWILPTLLYRSCFHIICLVGTISLACNDMTPEQMATNVNCSSPERHTRSYDYM
FGF-9                                          MAPLGEVGNYFGVQDAVPFGNVPVLPVDSPVLLSDHLGQSEAGGLPRGPAVTDLDH

AIGF         SRRLIRTYQLYSRTS-GKHVQVLANKRINAMAEDGDPFAKLIVETDTFGSRVRVRGAETGLYICMNKKGKLIAKSNGKGK
bFGF         PGHFKDPKRLYCKNG-GFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGV-VSIKGVCANRYLAMKEDGRLLA-SKCVTD
aFGF         PGNYKKPKLLYCSNG-GHFLRILPDGTVDGTRDRSDQHIQLQLSAESVGE-VYIKSTETGQYLAMDTDGLLYG-SQTPNE
INT-2        LGGAPRRRKLYCATK--YHLQLHPSGRVNGSLENSAYSI-LEITAVEVGI-VAIRGLFSGRYLAMNKRGRLYA-SEHYSA
HST/kFGF     LLGIKRLRRLYCNVGIGFHLQALPDGRIGGAHADTRDSL-LELSPVERGV-VSIFGVASRFFVAMSSKGKLYG-SPFFTD
FGF-5        WSLGARTGSLYCRVGIGFHLQIYPDGKVNGSHEANMLSV-LEIFAVSQGI-VGTRGVFSNKFLAMSKKGKLHA-SAKFTD
FGF-6        LVGIKHQRRLYCNVGIGFHLQGLPDGRISGTHEENPYSL-LEISTVERGV-VSLFGVRSALFVAMNSKGRLYA-TPSFQE
KGF          EGGDIRVRRLFCRTQW-Y-LRIDKRGKVKGTQEMKNNYNIMEIRTVAVGI-VAIKGVESEFYLAMNKEGKLYA-KKECNE
FGF-9        LKGILRRRQLYCRTG--FHLEIFPNGTIQGTRKDHSRFGILEFISIAVGL-VSIRGVDSGLYLGMNEKGELYG-SEKLTQ

AIGF         DCVFTEIVLENNYTALQNAKYEG----------------WYMAFTRKGRPRKG--SKTRQHQREVHFMKRLPRGHHTTEQ
bFGF         ECPFFERLGSNNYNTYRSRKYTS----------------WYVALKRTGQYKLG--SKTGPGQKAILFLPMSAKS
aFGF         ECLFLERLEENHYNTYISKKHAEKN----------------WFVGLKKNGSCKRG--PRTHYGQKAILFLPLPVSSD
INT-2        ECEFVERIHELGYNTYASRLYRTVSSTPGARRQPSAERLWYVSVNGKGRPRRGFKTRRT--QKSSLFLPRVLDHRDHEMV
HST/kFGF     ECTFKEILLPNNYNAYESYKYPG----------------MFIALSKNGKTKKG--NRVSPTMKVTHFLPRL
FGF-5        DCKFRERFQENSYNTYASAIHRTEKTGRE----------WYVALNKRGKAKRGCSPRVKPQHISTHFLPRFKQSEQPELS
FGF-6        ECKFRETLLPNNYNAYESDLYQG----------------YYIALSKYGRVKRG--SKVSPIMTVTHFLPRI
KGF          DCNFKELILENHYNTYASAKWTHNGGE------------NFYALNQKGIPVRGKKTKK---QKTAHFLPMAIT
FGF-9        ECVFREQFEENWYNTYSSNLYKHVDTGRR----------YYVALNKDGTPREG--TRTKRHQKFTHFLPRPVDPDKVPEL

AIGF         SLRFEFLNYPPFTRSLRGSQRTWAPEPRALAQLLPTQ
INT-2        RQLQSGKPRPPGKGVQPRRRRQKQSPDNLEPSHVQASRLGSQLEASAH
FGF-5        FTVTVPEKKNPPSPIKSKIPLSAPRKNTNSVKYRLKFRFG
FGF-9        YKDILSQS
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Fig. 2. Amino acid alignment of AIGF and its comparison with those of the other FGF family proteins. Amino acid alignments are depicted using a single letter code. Shaded regions indicate identical alignments among these proteins.

function-regulatory mechanism of this 100 k protein should be clarified by future experiments.

Although AIGF was identified as an obligatory intermediate in a process of androgen-induced growth, it remained to be determined whether or not additional androgen-regulated gene expression is required for eliciting growth. In this relation, repeated addition of partially purified AIGF into serum-free medium exhibited only transient growth-response with subsequent loss of responsiveness [24]. In addition, thyroid hormone has been observed to inhibit androgen-induced growth without affecting AIGF expression [25]. These results might suggest that an additional factor is definitely required for androgen-induced growth. To confirm this, AIGF expression vector was transfected into SC 3 cells to isolate the stable transfectants. All transfectants examined fail to grow in an androgen-independent manner and androgen stimulated the growth of these transfectants [26]. When stimulated with serum instead of androgen, however, these transfectants are much more rapidly converted into androgen-independent cells compared with the wild SC 3 cells. These observations would suggest that constitutive expression of AIGF alone is not sufficient for but facilitates the conversion from androgen-dependent to -independent phenotype.

CLINICAL IMPLICATION OF THE CURRENT FINDINGS ON SC 3 CELLS

These results on SC 3 cells summarized in this review seem to contain important implications on medical therapies against human hormone-dependent cancer. Firstly, hormone therapies should be carried out even though the tumor mass is contaminated with hormone-independent cells. It might be possible that the growth of hormone-independent cells is retarded or discontinued by hormone therapies through interrupting the synthesis of the growth factor in hormone-dependent cells. This is conceivable especially when hormone therapy is initiated at the early clinical stage. Since almost all human prostate carcinomas have been known to consist of heterogenous cell populations in terms of androgen receptor expression [27], the molecular talk between androgen-dependent and -independent cells seems to be important. The present finding would provide us with the theoretical basis for the clinical results that prostate carcinoma consisting of a mixture of cells with heterogenous androgen receptor expression is able to be controlled by hormone therapies. Secondly, the growth factor has been clearly demonstrated as an obligatory intermediate in the process of hormone-dependent growth of transformed target cells. Some growth factors have been implicated to be involved in hormone-dependent growth of various experimental models such as MCF-7 cells [28]. To conclude this, however, we have to experimentally confirm several points: (i) that the expression of the growth factor is under the strict control of hormonal steroid; (ii) that the functional blockade of the induced growth factor abolishes hormone-dependent growth; and (iii) that a receptor for hormone-inducible growth factor must be expressed on the cell-surface of transformed cells. To our knowledge, AIGF is the first example which completely satisfied these criteria. Thus, the growth factor and its receptor seem to be the important therapeutic targets in hormone-dependent cancer.

Our studies reveal that suramin is a possible candidate for this purpose. Actually, Winnan *et al.* reported impressive subjective and objective responses in their patients with relapsed prostate cancer [29]. This is a valuable clinical observation, although suramin is known to have relatively severe toxicity and to exhibit only poor response in patients with heavily pretreated relapsed prostate cancer [30]. When the action mechanism of suramin is considered in relapsed prostate cancer, the paracrine mechanism should be taken into consideration. Even though growth factor synthesis in transformed cells is blocked by androgen ablation therapies, the other growth factor with a similar biological potency can be constitutively secreted from parenchymal cells infiltrated into the tumor mass. The important role of various growth factors in regulation of prostate-specific gene expression has been also reported in human prostate cancer cells [31]. In the case of the SC 3 cell system, the FGF family of proteins listed in Fig. 2 have a biological potency not identical with but similar to AIGF. This consideration might provide us with the theoretical background for the use of suramin for relapsed prostate cancer. In addition, suramin is also able to inhibit the expression of FGFR 1 mRNA [11]. Since androgen-regulated gene product(s) other than AIGF have also been found to be required to induce the growth of SC 3 cells and many relapsed prostate cancer cells have been reported to retain the functional androgen receptor, androgen ablation therapies should be continued even after suramin administration is initiated to patients with relapsed prostate cancer. In view of the well-known clinical evidence that almost all patients with prostate cancer eventually escape the control of the first-line endocrine therapy and relapse after a median time of 12–18 months and respond poorly to conventional cytotoxic drugs [32], this suramin plus androgen ablation therapy might be considered as the possible second-line therapy. In this relation, development of a new drug with suramin-like potency but with reduced toxicity is definitely required.

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