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Facilitation of Autonomous Phenotype Acquisition in Androgen-dependent Shionogi Carcinoma 115 Cells by Transfection of Androgen-induced Growth Factor Expression Vector

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Androgen-induced growth factor (AIGF) is an autocrine growth factor for androgen-dependent SC-3 cells, which is induced by androgen stimuli. To elucidate the mechanism of the progression from hormone-dependent to -independent tumor, we transfected an expression vector of cDNA encoding AIGF into SC-3 cells and established a stable transfectant (A1) expressing AIGF. A1 cells showed enhanced DNA synthesis. This enhanced DNA synthesis was blocked by exposing the cells to AIGF antisense oligonucleotides, heparin, or suramin, indicating that enforced AIGF expression is responsible for the increase in DNA synthesis. However, A1 cells did not grow in serum-free medium unless stimulated with androgen. Recloning from A1 cells in semi-solid agar supplemented with fetal calf serum but without androgen quickly generated an autonomous subline that was able to grow rapidly in the serum-free medium irrespective of androgen stimulus. Mock-transfected SC-3 cells failed to form any colony under identical conditions. These results suggest that stable expression of AIGF alone is not sufficient for, but facilitates the conversion of SC-3 cells from androgen-dependent to -independent phenotype.

Key words: Androgen-dependent cancer — Growth factor — Stable transfectant — Androgen dependency

Considerable evidence indicates that sex hormones are important for the growth of hormone-dependent cancers such as breast cancer and prostate cancer.[1,2] These sex hormones exert their growth-stimulatory actions through complexing with the cognate nuclear receptor, which in turn activates a variety of hormone-regulated genes.[3,4] The role of sex hormones in the growth control of breast cancer and prostate cancer is also confirmed by the clinical observation that endocrine therapies are effective in treating some of these tumors.[5,6] Even though these tumors exhibit a transient response to such therapies, they often regrow in a hormone-independent manner, ultimately resulting in the patients' death.[7,8] Thus, it is critically important to know the mechanism underlying the progression from hormone-dependent to -independent cancer. In order to understand this mechanism, it seems to be prerequisite to clarify the molecular mechanism of hormone-enhanced cancer growth.

Human breast cancer cells such as MCF-7 cells have been extensively used to examine the role of sex hormones in malignant cells. Estrogenic stimuli have been observed to result in secretion of TGF-α[5] and insulin-like growth factor-I from MCF-7 cells.[9] On the basis of these observations, estrogen-induced growth factors have been proposed to be the obligatory component in estrogen-enhanced growth of breast cancer.[10] If one of these growth factors plays a pivotal role in the growth of estrogen-dependent cancer, one may speculate that constitutive expression of the particular growth factor is likely to be the mechanism underlying the progression to hormone-independent cancer. Actually, conditioned medium collected from estrogen-stimulated MCF-7 cells has been found to confer upon MCF-7 cells the ability to grow in estrogen-unstimulated nude mice.[11] However, gene transfection and stable expression of TGF-α failed to induce the hormone-independent phenotype of MCF-7 cells.[12] Unfortunately, these results do not exclude a possible role of constitutive growth factor expression in acquiring the hormone-dependent phenotype, since MCF-7 cells secrete multiple growth factors in response to estrogen stimuli. Moreover, antibody against epidermal growth factor receptor has been reported to inhibit TGF-α-dependent, but not estrogen-dependent growth.[13] Thus, the molecular mechanism underlying the estrogen-dependent growth of MCF-7 cells remains obscure, implying that MCF-7 cells are not suitable for investigating

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[5] The following abbreviations are used: TGF-α, transforming growth factor-α; AIGF, androgen-induced growth factor; bFGF, basic fibroblast growth factor; MEM, minimum essential medium; FCS, fetal calf serum; DCC, dextran-coated charcoal; HMB medium, Ham F-12: MEM (1:1, v/v) containing 0.1% (w/v) essentially fatty acid-free bovine serum albumin.

the role of growth factor expression in inducing the hormone-independent phenotype of cancer cells.

SC-3 cells cloned from androgen-dependent mouse mammary carcinoma (Shionogi carcinoma 115) are remarkably growth-stimulated by androgen in a defined medium.[14] This androgen-enhanced growth has been postulated to be mediated through induction of a heparin-binding growth factor. This growth factor (androgen-induced growth factor: AIGF) is composed of 215 amino acids with approximately 30% homology with bFGF.[15] Inhibition of the translation of AIGF mRNA by specific AIGF antisense oligonucleotides is accompanied with complete block of androgen-induced DNA synthesis but not bFGF-induced DNA synthesis, indicating that androgen-induced DNA synthesis is strictly dependent upon AIGF.[16] These results can be interpreted as an indication that SC-3 cells are suitable for investigating the role of AIGF expression in the process of the progression from hormone-dependent to -independent cancer. In addition, establishment of SC-3 cells stably expressing AIGF enables us to address the important question of whether some additional androgen-regulated gene product(s) other than AIGF is required for androgen-induced growth of malignant cells. Thus, in this study, we transfected the AIGF expression vector into SC-3 cells and examined the growth response of the transformed cells to androgen stimuli.

MATERIALS AND METHODS

Cell line and establishment of stable transfectants SC-3 cells cloned from mouse mammary carcinoma (Shionogi carcinoma 115) were maintained in MEM supplemented with 2% FCS, 10^{-8} *M* testosterone and 2 m*M* glutamine. To construct the expression vector encoding AIGF, cDNA of AIGF was inserted into pcDLSRα296.[16] The expression vectors of AIGF (18 μg) and neomycin-resistance gene (2 μg) were co-transfected into SC-3 cells preplated at a density of 5×10^5 per 10-cm dish by the calcium precipitation method.[16, 17] As a control, the expression vector of human bFGF[18] was also employed. Mock-transfection [2 μg of neomycin-resistance gene and 18 μg of pcDLSRα296 vector (no insertion)] was also carried out. These cells were cultured in MEM supplemented with 10% FCS, 10^{-8} *M* testosterone and 1 mg/ml of G418 (Geneticin: Gibco Laboratories, Detroit, MI) for 14 days. The foci formed were clonally isolated. In the present studies, representative transfectants expressing AIGF and bFGF were used and designated as A1 cells and B1 cells, respectively. To examine the effect of androgen withdrawal on hormone-dependency, A1 cells were subjected to an additional cloning in semi-solid agar supplemented with MEM containing 10% DCC-treated FCS (no androgen).[16] After 14 days of culture, one of the colonies was isolated and designated as A1b cells.

Cell growth assays The cells (10^4/well) were plated on 24-well plates in 2% DCC-treated FCS-MEM unless otherwise specified. Twenty-four hours later, the medium was changed to HMB medium supplemented with test compounds (day 1). Thereafter, the medium was changed every other day. The cell number was counted on the indicated day. To determine the DNA synthesis, the cells were plated in 2% DCC-treated FCS-MEM on 96-well plates at a density of 10^4 cells per well. After 24 h culture, the cells were washed twice with HMB medium, incubated in HMB medium for 24 h and then stimulated with test compounds. After 24 h stimulation, the cells were pulse-labeled with [^{3}H]thymidine (Amersham Japan, Tokyo) for 2 h.[19] Each assay was performed in triplicate. The sequences of AIGF antisense and sense phosphorothioate oligonucleotides used in the present study, designed to encompass the translation initiation site, were as follows: 5′-GCGGGGGCTGCCCAT3′ (antisense); 5′ATGGGCAGCCCCCGC3′ (sense). The concentration (0.5 μM) of these oligonucleotides used in the present study was found to be optimum to obtain their specific effects.[16]

Northern blot analysis Total cellular RNA (10 μg/lane) extracted from each cell line was electrophoresed to carry out Northern blot analysis using AIGF cDNA as a hybridization probe.[20] The filter was rehybridized with β-actin probe.

Statistics All values presented here were means$\pm$SE obtained by triplicate assays.

RESULTS

Establishment of stable transfectant expressing AIGF or bFGF To establish a stable transfectant expressing AIGF, the expression vectors of AIGF and neomycin-resistance gene were co-transfected into SC-3 cells and the transformed cells were cultured in the presence of G418. A representative transfectant expressing AIGF was designated as A1 cell line and used for the following experiments. As shown in Fig. 1, the unstimulated A1 cells were observed to express AIGF mRNA. Testosterone stimulation resulted in the elevation of the AIGF mRNA content in A1 cells, indicating that the endogenous AIGF gene can be activated by androgen. In contrast, AIGF mRNA in mock-transfected cells was undetectable, but was induced by the stimulation with testosterone. These results indicated that a stable transfectant expressing AIGF had been established. A similar procedure revealed that B1 cells are capable of expressing bFGF mRNA (data not shown).

Effects of compounds modulating AIGF activity on DNA synthesis of stable transfectants The basal level of DNA

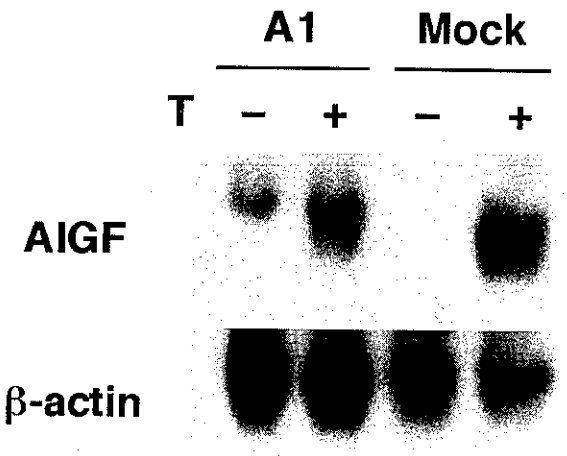

Fig. 1. Northern blot analyses of AIGF mRNA in mock-transfected cells (Mock) and A1 cells treated with or without androgen. The cells were unstimulated or stimulated with 10^{-8} M testosterone (T) for 24 h. The RNAs (10 μg/lane) extracted from these cells were electrophoresed to measure their AIGF mRNA contents by Northern blot analysis.

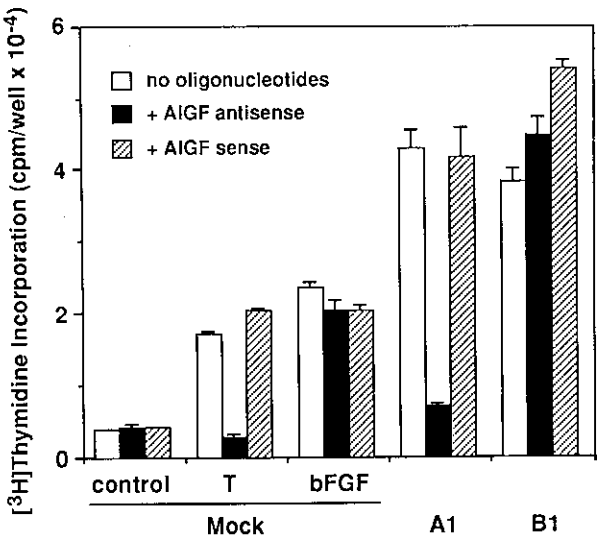

Fig. 2. Effects of AIGF antisense oligonucleotides on DNA synthesis in mock-transfected (Mock), A1 or B1 cells. The mock-transfected cells unstimulated (control) or stimulated with 10^{-8} M testosterone (T) or 1 ng/ml bFGF were treated with 0.5 μM AIGF antisense or sense oligonucleotides. A1 or B1 cells were also treated with these oligonucleotides without any stimulants. After 24 h, DNA synthesis was measured as described in "Materials and Methods."

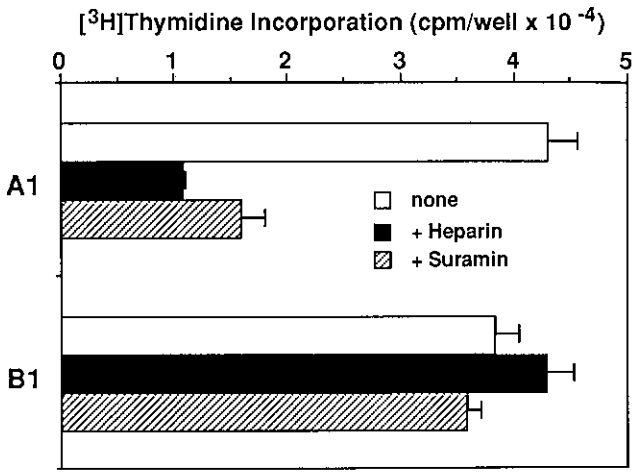

Fig. 3. Effects of heparin or suramin on DNA synthesis in A1 or B1 cells. A1 or B1 cells were treated with 10 μg/ml heparin or 100 μM suramin for 24 h, and then DNA synthesis was measured as described in "Materials and Methods."

synthesis in A1 or B1 cells was much higher than that of mock-transfected cells without any stimulants. Testosterone-, but not bFGF-, enhanced DNA synthesis in mock-transfected cells was inhibited by 0.5 μM AIGF antisense oligonucleotides, suggesting that this antisense probe is a useful tool to inhibit specifically the AIGF-dependent events (Fig. 2). The DNA synthesis in A1 cells, but not in B1 cells, was inhibited by AIGF antisense oligonucleotides. AIGF sense oligonucleotides did not affect the DNA synthesis in any cell line. These results indicate that the enforced expression of AIGF is responsible for the accelerated DNA synthesis in A1 cells. To confirm this, we examined the effects of suramin and heparin, which have been demonstrated to inhibit the activity of AIGF but not bFGF,[21,22] on the DNA synthesis in these cell lines. Both heparin (10 μg/ml) and suramin (100 μM) inhibited the DNA synthesis of A1 cells, but not B1 cells, under the present experimental conditions (Fig. 3).

Next, the concentration-dependent effect of testosterone on the DNA synthesis in A1 cells was examined (Fig. 4). The DNA synthesis in mock-transfected cells was markedly enhanced by testosterone in a concentration-dependent manner. On the other hand, testosterone did not further enhance the DNA synthesis in A1 cells, indicating that the AIGF expression in unstimulated A1 cells is sufficient to generate the maximum DNA synthesis.

Effects of androgen on the proliferation of A1 or mock-transfected cells Since the enhanced DNA synthesis in A1 cells was shown to be mediated through the constitutive expression of the transfected AIGF cDNA, it seemed likely that A1 cells would grow autonomously even without androgen. To address this, A1 cells were plated at various cell densities and were cultured in HMB medium supplemented with or without testosterone (Fig. 5).

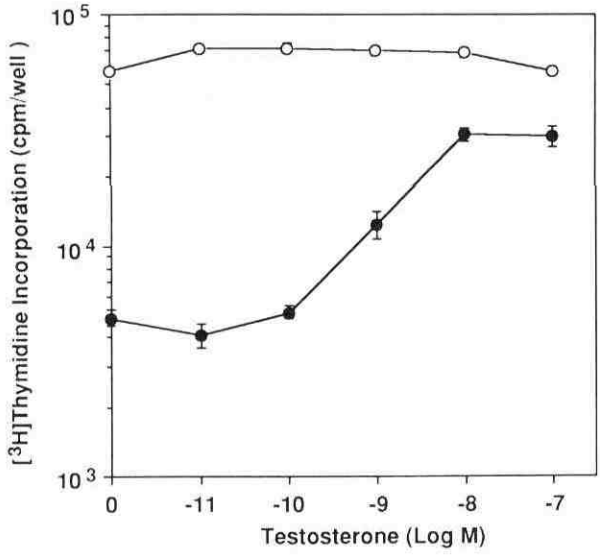

Fig. 4. Effect of testosterone on DNA synthesis in mock-transfected or A1 cells. The mock-transfected (closed symbols) or A1 cells (open symbols) were stimulated with various concentrations of testosterone for 24 h and DNA synthesis was measured as described in "Materials and Methods." Where error bars are not shown, they did not exceed the size of the symbol.

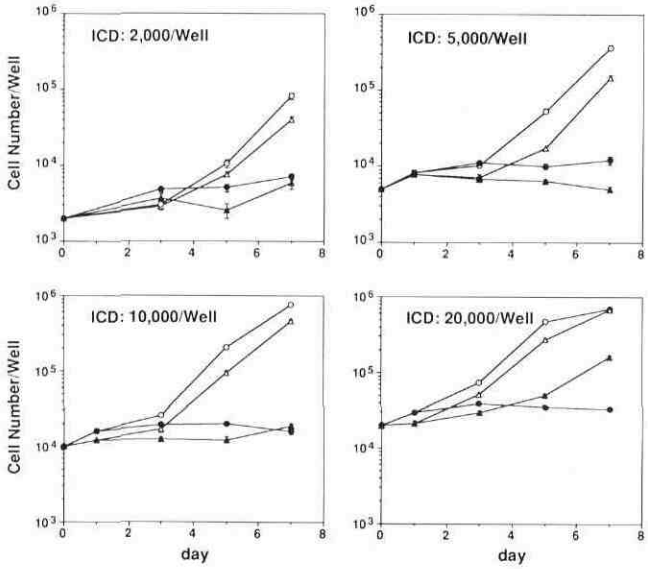

Fig. 5. Effects of testosterone on the proliferation of mock-transfected or A1 cells. The mock-transfected (●, ○) or A1 (△, ▲) cells were plated at various initial cell densities (ICDs) as indicated on each panel, and then unstimulated (●, ▲) or stimulated (○, △) with 10^{-8} *M* testosterone for the indicated periods. The cell number was measured. Where error bars are not shown, they did not exceed the size of the symbol.

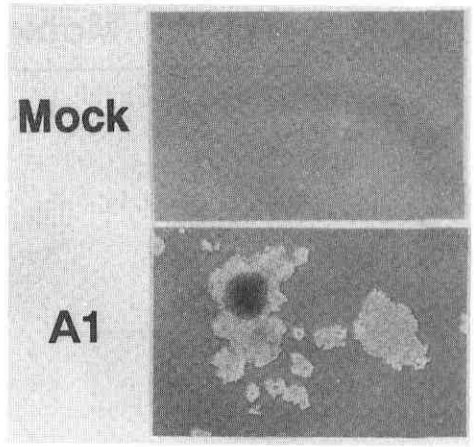

Fig. 6. Photographic illustration of the ability for A1 cells to form colonies in semi-solid agar. The mock-transfected (Mock) or A1 cells were suspended in 0.3% Noble agar in 10% FCS-MEM at a cell density of 10^3 per 3 ml. This solution was over-layered on 6 ml of 0.5% Noble agar in 10% FCS-MEM in a 6-cm dish. After 14 days of incubation without medium change, the colonies formed were photographed (×100).

Unexpectedly, A1 cells unstimulated with androgen did not grow, and their cell numbers remained at the initial level up to day 7. These results were similar to those for androgen-unstimulated mock-transfected cells. In contrast, both A1 and mock-transfected cells were remarkably growth-stimulated by testosterone. These results indicate that the enforced expression of AIGF is insufficient to induce the androgen-independent phenotype in SC-3 cells.

Rapid induction of androgen-independent phenotype in A1 cells by re-cloning in the absence of androgen Prolonged culture of Shionogi carcinoma 115 cells without androgen was shown to confer the androgen-independent phenotype.[23, 24] Thus, we attempted to re-clone A1 cells by culture in semi-solid agar containing 10% DCC-treated FCS alone (no androgen) for 14 days as described previously.[16] In this condition, mock-transfected cells failed to form any colony while A1 cells formed many colonies (Fig. 6). One isolated subclone was termed A1b cell line and used for the following experiments. Total RNAs extracted from androgen-unstimulated and -stimulated A1b cells were subjected to Northern blot analysis (Fig. 7A). The unstimulated A1b cells were found to express a comparable level of AIGF mRNA to that of unstimulated A1 cells. The level was not elevated further in response to androgen stimulus, in contrast to that obtained by using A1 cells. To examine the biological consequence of AIGF expression, the effect of AIGF antisense oligonucleotides on the DNA synthesis in A1b

cells was examined (Fig. 7B). The DNA synthesis in A1b cells was inhibited by AIGF antisense oligonucleotides, indicating that AIGF expression was responsible for the enhanced DNA synthesis in A1b cells.

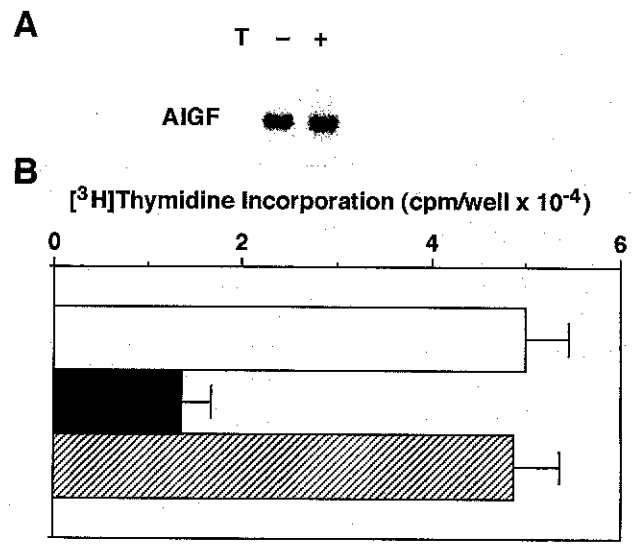

Fig. 7. Northern blot analysis of AIGF mRNA expression in A1b cells and the effect of AIGF antisense on their DNA synthesis. The RNA extracted from A1b cells that had been unstimulated or stimulated with 10^{-8} *M* testosterone (T) for 24 h was treated as described in the legend to Fig. 1 (panel A). The effects of AIGF antisense (closed column) or sense (hatched column) oligonucleotides on the DNA synthesis in A1b cells were also examined as described in the legend to Fig. 2 (panel B). Open column; no oligonucleotides.

Effects of androgen on DNA synthesis and proliferation of A1b cells To examine the effect of androgen on the DNA synthesis, A1b cells were stimulated with androgen. The DNA synthesis of unstimulated A1b cells was observed to be high, and androgen failed to enhance their DNA synthesis further (Fig. 8A). In addition, A1b cells proliferated well without androgen stimulus and their proliferation rate was not elevated by addition of testosterone at any concentration examined (Fig. 8B). Thus, A1b cells can be categorized as an autonomous cell line. Similar results were obtained with two additional subclones (data not shown).

DISCUSSION

AIGF is an essential component in an androgen-inducible autocrine loop in SC-3 cells, since AIGF antisense oligonucleotides can completely block the androgen-enhanced DNA synthesis.[16] The present study also showed that the DNA synthesis enhanced by stable expression of AIGF cDNA was inhibited by AIGF antisense oligonucleotides, heparin or suramin, indicating that the enhanced DNA synthesis in A1 cells is mainly attributable to the transfected AIGF cDNA. However, this enhanced DNA synthesis is not accompanied with anchorage-dependent growth. The possibility arises that the expression level of the transfected AIGF vector is quantitatively insufficient for supporting the cell proliferation. However, this seems unlikely because AIGF mRNA contents in both unstimulated A1 (androgen-dependent for cell proliferation) and A1b (androgen-independent) cells are similar, and DNA synthesis in both cell lines can

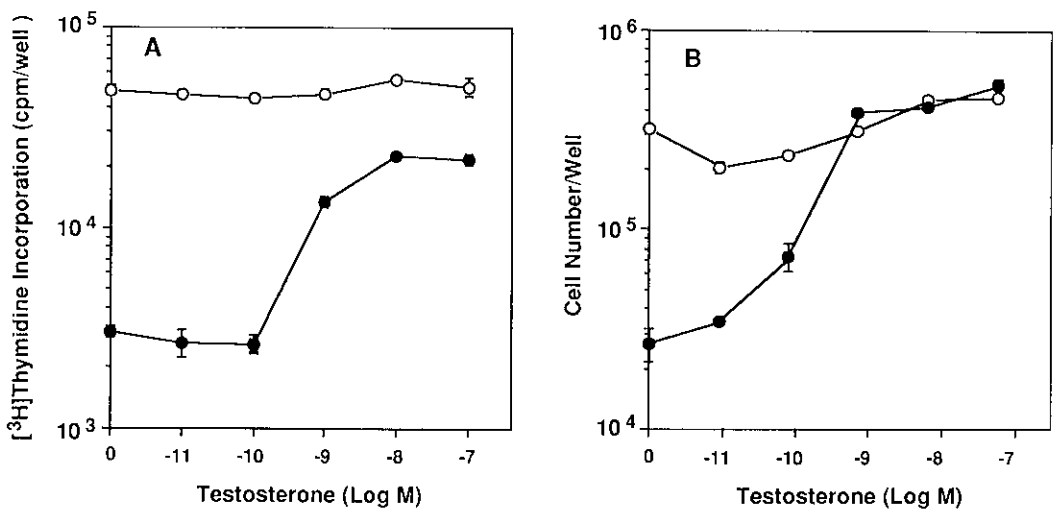

Fig. 8. Effect of testosterone on the DNA synthesis or proliferation of A1b cells. The DNA synthesis (panel A) and cell proliferation (panel B) of mock-transfected cells (closed symbols) or A1b cells (open symbols) were measured under the standard conditions, after stimulation with the indicated concentrations of testosterone for 1 day (panel A) or 6 days (panel B). Where error bars are not shown, they did not exceed the size of the symbol.

be blocked by AIGF antisense oligonucleotides. Furthermore, levels of [^{3}H]thymidine incorporation in unstimulated A1 and A1b cells are similar and are not further enhanced by androgen stimulus, suggesting that DNA synthesis in both cell lines is maximally stimulated by AIGF. Thus, we conclude that AIGF expression alone is insufficient for SC-3 cells to exhibit the androgen-independent phenotype.

The most noteworthy finding in the present study is that stable expression of AIGF cDNA confers upon SC-3 cells the ability to grow in semi-solid agar in an androgen-independent fashion. The wild-type SC-3 cells cannot grow in semi-solid agar, except in the presence of androgen. Various expression vectors encoding growth-regulating genes such as TGF-α have been transfected into MCF-7 cells.[12] However, phenotypic alteration in response to transfection of these genes has not been observed. Thus, the present study is the first demonstration that transfection of an expression vector encoding a growth-regulating gene alters the growth behavior of hormone-dependent cancer cells in semi-solid agar.

A1 cells showed anchorage-independent growth but not anchorage-dependent growth. This difference may be due to the fact that FCS was used for the anchorage-independent growth experiments. In fact, FCS can reduce the androgen dependency of A1 cells on culture dishes (data not shown). AIGF might promote the cell proliferation in concert with a component(s) in FCS. These observations are consistent with our speculation that AIGF is an obligatory component in androgen-inducible autocrine loop, but an additional component is required for the continuous growth of SC-3 cells.[25] In connection with this, we recently found that unsaturated fatty acid promotes the growth of SC-3 cells stimulated with bFGF or AIGF under serum-free conditions (S. Kasayama *et al.*, submitted for publication).

A1 cells were observed to be rapidly converted from androgen-dependent to -independent phenotype by culture in androgen-free semi-solid agar. To obtain an autonomous subline from SC-3 cells, prolonged culture (more than 10 passages) in androgen-free but serum-containing medium is required.[23, 24] Therefore, the present data can be interpreted as an indication that transfection of AIGF expression vector can facilitate the progression of SC-3 cells to autonomous phenotype. Time-consuming and complicated procedures have been employed to establish a tamoxifen-independent subline from MCF-7 cells.[26] These procedures might be expected to elicit mutations of growth-related as well as -unrelated genes. Multiple gene alterations would make it difficult to isolate the gene that is actually involved in the progression from hormone-dependent to -independent cancer. On the other hand, the rapid and reproducible procedure described here for obtaining an autonomous subline should minimize gene alterations. Thus, the cell line established in the present study might be a useful tool to isolate the gene(s) involved in the progression from hormone-dependent to -independent cancer by means of procedures such as a subtraction hybridization technique. Work in our laboratory is currently directed toward isolation of this gene.

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