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Unsaturated Fatty Acids Are Required for Continuous Proliferation of Transformed Androgen-dependent Cells by Fibroblast Growth Factor Family Proteins[1]

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ABSTRACT

Increase in dietary fat intake has been reported to be associated with progression of hormone-dependent cancers. To explore its mechanism, we examined the effects of fatty acids on the growth of androgen-dependent SC-3 cells cloned from mouse mammary cancer (Shionogi carcinoma 115). Their androgen-dependent growth was potentiated by linoleic acid in the defined medium. The effect of linoleic acid on fibroblast growth factor (FGF)-dependent growth was also addressed because androgen had been demonstrated to exert its mitogenic activity on SC-3 cells through an induction of the unique FGF family protein termed as androgen-induced growth factor. Exposure of SC-3 cells to basic FGF or androgen-induced growth factor exhibited only transient growth response. However, simultaneous addition of linoleic acid to the medium sustained the proliferation of FGF-stimulated, but not FGF-unstimulated, cells, although linoleic acid did not exert the significant effect on the process of S-phase entry of basic FGF-stimulated cells. Palmitoleic acid and oleic acid appeared to exert the actions similar to linoleic acid, while stearic acid was without any effect. Neither cyclooxygenase inhibitor nor 5-lipoxygenase inhibitor could block the growth-promoting ability of linoleic acid. Linoleic acid also enhanced their anchorage-independent growth in the presence of basic FGF. These results indicate that these unsaturated fatty acids play a role in sustaining the proliferation of FGF-stimulated SC-3 cells.

INTRODUCTION

The incidence of hormone-dependent prostate and breast cancers has been shown to be positively associated with dietary fat intake (1, 2). The strength of the association with overall levels of fat in consumption increases with age and is strongest in the ages with peak cancer incidence rates. Reductions in cancer incidence rates resulting from a sustained 50% cut in the average consumption of fat in men and women of age 55–69 years were estimated to be 83% for prostate cancer and 61% for breast cancer (3). These epidemiological studies suggest that dietary lipids promote the progression of the hormone-dependent cancers.

In addition to the quantity of fat consumed, its FA[3] composition should be taken into consideration when the mechanism of fat intake-dependent increase in hormone-dependent cancers is considered. Most epidemiological studies implicate that high intake of fat of animal origin mainly composed of saturated rather than unsaturated FA is causally related to the incidence of breast cancer (4–6), although this dietary behavior may cause obesity, which in turn elevates serum hormone levels (7, 8). On the other hand, the increasing incidence of breast cancer has been suggested to be related to the sustained increase in vegetable oil consumption (principally n-6 unsaturated FAs) (9, 10). In addition, patients with prostate cancer have been reported to have a significantly higher concentration of oleic acid (18:1, n-6) in phospholipids from both plasma and prostatic tissue when compared with those with benign prostatic disease (11). In line with these results on human subjects, dietary fats high in LA (18:2, n-6) have been observed to enhance the growth (12, 13) and metastasis (14–17) of murine mammary carcinomas. However, these *in vivo* studies could not allow us to discuss the direct effect of unsaturated FA on cancer cells.

To investigate the direct effect of FAs on hormone-dependent cancers, the culture systems were definitely required. To do so, we needed to overcome two major obstacles: (*a*) many hormone-responsive transformed cells for culturing require the serum that contains unknown amounts of FAs; and (*b*) the molecular mechanism of hormone-dependent growth is largely obscure in many established cell lines. In this relationship, SC-3 cells cloned from mouse mammary carcinoma (Shionogi carcinoma 115) seemed to be quite suitable for addressing the effect of FAs on their growth since this cell line exhibits the remarkable growth-stimulatory response to androgen in the defined medium (18). This androgen stimulation results in a secretion of an autocrine heparin-binding growth factor (19), which in turn binds to FGF receptor 1 (20, 21). Thus, the ligand-induced activation of FGF receptor 1 plays a central role for the SC-3 cell growth. This consideration is further supported by the finding that both bFGF and aFGF can initiate the DNA synthesis of the SC-3 cells (22). We have recently cloned cDNA encoding the autocrine growth factor termed as AIGF, which is identified as a new member of FGF family proteins (23). Taking these advantages into consideration, we have examined the effects of FAs on the growth of the SC-3 cells unstimulated or stimulated with androgen or these growth factors.

MATERIALS AND METHODS

Materials. Bovine brain-derived aFGF and human recombinant bFGF were obtained from R&D Systems (Minneapolis, MN). Heparin-Sepharose was purchased from Pharmacia (Piscataway, NJ). BSA (essentially fatty acid-free), FAs, indomethacin, and testosterone were from Sigma Chemical Co. (St. Louis, MO). [*methyl*-^{3}H]thymidine (70–85 Ci/mmol) and Ampure SA column were from Amersham Japan (Tokyo, Japan). AA 861 was kindly supplied by Takeda Pharmaceutical Co. (Osaka, Japan). All other chemicals were of analytical grade.

Cell Growth Experiments. A clonal cell line from Shionogi carcinoma 115, SC-3, was established as described previously (18). The SC-3 cells were maintained in MEM supplemented with 2% FCS (Hyclone, Logan, UT) and 10^{-8} M testosterone, and were kept at 37°C in a humidified 5% CO_2 incubator. NIH3T3 cells, obtained from RIKEN (Saitama, Japan), were cultured in DMEM supplemented with 10% calf serum.

For the anchorage-dependent growth experiments, these cells were plated onto a 24-well plate (10^4 cells/well) in 1 ml MEM containing 2% DCC-treated FCS (18). On the following day (day 0), the medium was replaced with 1 ml HMB medium containing various test compounds. The serum-free medium was changed every other day. The viable cell number was counted on day 6, unless specified otherwise.

For the anchorage-independent growth experiments, the cells were suspended in 0.4% Noble agar (DIFCO Laboratories, Detroit, MI) in HMB

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[3] The abbreviations used are: FA, fatty acid; LA, linoleic acid; FGF, fibroblast growth factor; b/a FGF, basic/acidic FGF; AIGF, androgen-induced growth factor; BSA, essentially fatty acid-free bovine serum albumin; HMB, linoleic acid-free Ham's F-12:MEM (1:1, v/v) containing 0.1% BSA; DCC, dextran-coated charcoal.

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medium supplemented with 1% DCC-treated FCS and the test compounds at a density of 2×10^4 cells/5 ml. This solution (5 ml) was overlayered on 5 ml of 0.5% Noble agar in the above medium in 60-mm dish. These cells were incubated in 5% CO_2-95% air at 37°C for 14 days without medium change. Then the visible colonies formed were counted (21).

DNA Synthesis. [^{3}H]Thymidine incorporation into DNA of SC-3 cells was measured by the method as described (24). Briefly, the cells were plated onto a 96-well plate (5×10^3 cells/well) in 0.15 ml MEM supplemented with 2% DCC-treated FCS. On the following day, the cells were washed once with HMB medium and kept in HMB medium for 24 h. The quiescent cells were treated with test compounds for 24 h. Then the cells were pulse labeled with [^{3}H]thymidine (0.3 μCi/well) for 2 h to measure the DNA synthesis.

Partial Purification of AIGF. AIGF was extracted from the androgen-stimulated SC-3 cell culture (25). The SC-3 cells were stimulated with 10^{-8} M testosterone in HMB medium for 48 h. After the conditioned medium was taken out, the dish was extracted with 2 M NaCl, 10 mM HEPES (pH 7.5 at 20°C) and 0.01% BSA at 4°C for 1 min. This extracted solution was applied to an Ampure SA column to remove excess NaCl. Then AIGF was partially purified by means of a heparin-Sepharose column (26).

Statistical Analysis. All values presented here were means ± SE. When the significant difference was discussed, analysis of variance with a multiple range test was used.

RESULTS

Effects of FAs Exogenously Added into HMB Medium on Proliferation of SC-3 Cells. In view of the *in vivo* studies describing that LA (one of essential FAs)-enriched dietary fat enhanced the growth of rodent mammary tumors (12–14), an initial experiment was conducted to examine the effects of LA on anchorage-dependent growth of SC-3 cells. The growth experiment was performed in serum-free medium with or without LA-BSA conjugate at the final BSA concentration of 0.1%. As shown in Fig. 1, testosterone-unstimulated cells could not proliferate in HMB medium irrespective of the presence of LA. In the absence of LA, testosterone stimulation enhanced their proliferation whereas bFGF elicited only a transient growth response on day 2 without further increase in the cell yield at the later stimulation stage (Fig. 1*A*). These results were consistent with our previous observation (27). In contrast, simultaneous addition of LA in HMB medium at a concentration of 10 μg/ml (35 μM) allowed the continuous proliferation of the bFGF-stimulated cells (Fig. 1*B*). LA also enhanced the proliferation of testosterone-stimulated cells, although the effects appeared to be less remarkable than those on bFGF-stimulated cells. Next, the concentration-dependent effect of LA on bFGF (2 ng/ml, 0.11 nM)-stimulated SC-3 cell growth was examined. LA at concentrations ranging from 2.5 to 10 μg/ml was observed to elicit the significant enhancement of the cell proliferation in a dose-dependent manner (Fig. 2). In the following experiments, LA was used at a concentration of 10 μg/ml.

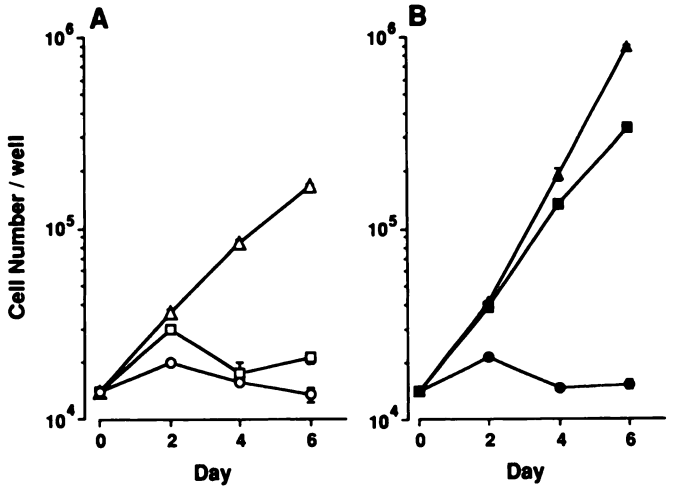

Fig. 1. Time course studies on LA effects on the growth of SC-3 cells stimulated with testosterone or bFGF. The cells were plated on triplicate wells as described in "Materials and Methods." On the following day (day 0), the medium was replaced with HMB medium supplemented with 10^{-8} M testosterone (△, ▲) or 2 ng/ml bFGF (□, ■) in the absence (*A*) or presence (*B*) of 10 μg/ml LA. The effect of LA on the growth of the cells unexposed to growth stimulants was also analyzed (○, ●). The cell number was counted on the indicated day. *Points*, mean; *bars*, SE.

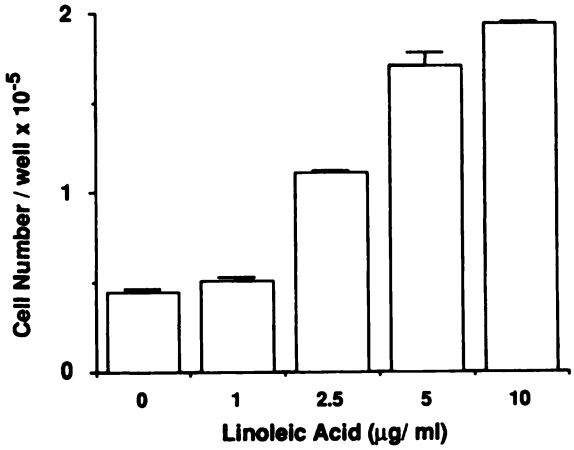

Fig. 2. Concentration-dependent growth promotion of LA on bFGF-stimulated SC-3 cell growth. The cells were plated on triplicate wells as described in "Materials and Methods." On the following day, the cells were stimulated with 2 ng/ml bFGF in the presence of various concentrations of LA. The cell number was counted on day 6. *Columns*, mean; *bars*, SE.

Table 1 *Effects of FAs on the growth of SC-3 cells stimulated with testosterone or bFGF*

The cells were plated on triplicate wells as described in "Materials and Methods," and then stimulated with 10^{-8} M testosterone or 10 ng/ml bFGF in the presence of 10 μg/ml FA. The unstimulated cells (no addition) were also cultured. The cell number was counted 6 days after the stimulation.

Treatment	Cell number × 10^4/ well
No addition	3.1 ± 0.3
LA (18:2, n-6)	3.4 ± 0.4
Testosterone	
Alone	19.8 ± 0.3
+ LA (18:2, n-6)	30.7 ± 0.7
bFGF	
Alone	4.5 ± 0.3
+ LA (18:2, n-6)	17.0 ± 1.1
+ Palmitoleic acid (16:1, n-6)	8.5 ± 0.4
+ Oleic acid (18:1, n-6)	9.7 ± 0.6
+ Linolenic acid (18:3, n-6)	0.3 ± 0.6
+ Arachidonic acid (20:4, n-6)	0.4 ± 0.1
+ Stearic acid (18:0)	3.1 ± 0.5

Since the growth-promoting ability of LA on bFGF-stimulated SC-3 cells became evident, the effects of other FAs on the SC-3 cell growth were examined (Table 1). Among FAs examined, LA was found to be most potent in promoting the proliferation of bFGF-stimulated SC-3 cells. Palmitoleic (16:1, n-6) and oleic (18:1, n-6) acids also enhanced their proliferation to a lesser degree. However, the saturated FA, stearic acid (18:0), failed to show any significant effect. Desaturated metabolites of LA such as linolenic (18:3, n-6) and arachidonic (20:4, n-6) acids exhibited the cytocidal action on the bFGF-stimulated cells. These results suggest that these mono- or diunsaturated FAs exert their growth-promoting action without conversion into prostaglandins and leukotrienes. To confirm this possibility, the effect of cyclooxygenase inhibitor (indomethacin) or 5-lipoxygenase inhibitor (AA 861) on the LA action was studied. As shown in Table 2, neither indomethacin nor AA 861 could affect the

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growth-promoting ability of LA on the bFGF-stimulated cells, even at the high concentrations.

Since testosterone is known to induce the morphological changes of SC-3 cells from polygonal to spindle shape (18, 19), the effect of LA on the morphology of the cells was addressed (Fig. 3). The SC-3 cells stimulated with testosterone showed the spindle shape irrespective of the presence of LA. On the other hand, bFGF as well as aFGF stimulated the SC-3 cell proliferation and induced the cells to spindle shape only in the presence of LA.

Effect of LA on Anchorage-independent Growth of SC-3 Cells. Since anchorage-independent growth has been considered to be an important phenotype of transformed cells, the ability of SC-3 cells to form a colony in a semisolid agar was investigated. In the absence of FCS, the cells could not form any colony, although the cells were stimulated with both testosterone and LA (data not shown). In the presence of 1% DCC-treated FCS, however, the SC-3 cells were able to form colonies in response to testosterone treatment. The concentration of LA in 1% DCC-treated FCS used here was approximately 0.17 μg/ml, analyzed by gas chromatography-mass spectrometry (28). Thus, the effect of FCS-derived LA is considered to be negligible in this experimental condition (Fig. 2). LA was observed to potentiate the androgen-induced colony formation, whereas LA alone was unable to form colonies (Table 3). In contrast to the androgen-stimulated cells, bFGF alone failed to provide SC-3 cells with the ability to form colonies. However, simultaneous exposure of the cells to bFGF and LA resulted in a colony formation, again indicating that LA plays an essential role for the SC-3 cells to exhibit the transformed phenotype.

Lack of the Ability of LA to Stimulate the S-Phase Entry of SC-3 Cells. Next, the effect of LA on the DNA synthesis of SC-3 cells was examined. After being plated in MEM with 2% DCC-treated FCS and kept in the serum-free medium (HMB) for 24 h, the cells were stimulated with testosterone or bFGF in the presence or absence of LA. As shown in Fig. 4, the DNA synthesis of the SC-3 cells unstimulated or stimulated with testosterone or bFGF was not affected by LA. The results suggest that LA cannot promote the first cell cycle from G_0-G_1 to S-phase in this experimental condition.

Table 2 *Effects of cyclooxygenase inhibitor (indomethacin) or lipoxygenase inhibitor (AA 861) on the growth of SC-3 cells exposed to bFGF and LA*

The cells were plated on triplicate wells as described in "Materials and Methods," and then stimulated with 10 ng/ml bFGF and 10 μg/ml LA in the presence or absence (control) of the indicated concentrations of the inhibitors. The cell number was counted 6 days after the treatment.

Treatment	Cell number (% of control)
Control	100 ± 7
Indomethacin	
10^{-8} M	97 ± 6
10^{-6} M	109 ± 9
AA 861	
10^{-8} M	90 ± 4
10^{-6} M	91 ± 6

Table 3 *Effect of LA on anchorage-independent growth of SC-3 cells stimulated with testosterone or bFGF*

The cells (2×10^4 cells/60-mm dish) were dispersed in semisolid agar supplemented with 10^{-8} M testosterone or 10 ng/ml bFGF in the presence or absence of 10 μg/ml LA. All media contained 1% DCC-treated FCS. The number of visible colonies was counted on day 14.

Treatment	No. of colonies/dish
No addition	0
LA	0
Testosterone	
Alone	1,300 ± 80
+ LA	3,300 ± 340
bFGF	
Alone	0
+ LA	3,800 ± 250

Growth Factor and Target Cell Specificity of LA Action. Since AIGF has been known to be an obligatory but not sufficient component in androgen-stimulated autocrine loop in SC-3 cells (21, 27), the effect of LA on AIGF-dependent growth of the SC-3 cells was examined. As shown in Fig. 5*A*, AIGF alone was not able to promote the proliferation of the SC-3 cells in the serum-free conditions. However, AIGF was able to promote their proliferation in the presence of LA. These results were similar to those on bFGF-stimulated SC-3 cells.

We also addressed whether the effects of LA could be observed in other FGF target cells. In Fig. 5*B*, NIH3T3 cells were exposed to bFGF alone or in combination with LA in HMB medium. The results showed that bFGF promoted the proliferation of NIH3T3 cells only in the presence of LA. LA alone did not exert any effects on NIH3T3 cell proliferation.

DISCUSSION

The present study has clearly demonstrated that mono- or diunsaturated FAs are able to support the anchorage-dependent and anchorage-independent growth of SC-3 cells stimulated with FGF family proteins. To our knowledge, this is the first report demonstrating the synergistic effect of LA and FGF on the growth of FGF target cells. FGF family proteins can promote the S-phase entry from the G_0-G_1 phase in our experimental condition. Since LA failed to modulate the FGF-dependent S-phase entry, these unsaturated FAs appear not to target the signal pathway prior to cell division of FGFs. Rather, it is suggested that LA exerts its growth-supporting action through promoting the cell cycle of FGF-primed SC-3 cells. LA might be a precursor needed for some structural aspect of the cell cycle. In addition, this synergistic effect was observed also in NIH3T3 cells, suggesting that LA has a general role in promoting the proliferation of FGF-stimulated cells.

The evidence for the important role of lipids in modulating cell proliferation has been accumulated. Phospholipase C-catalyzed

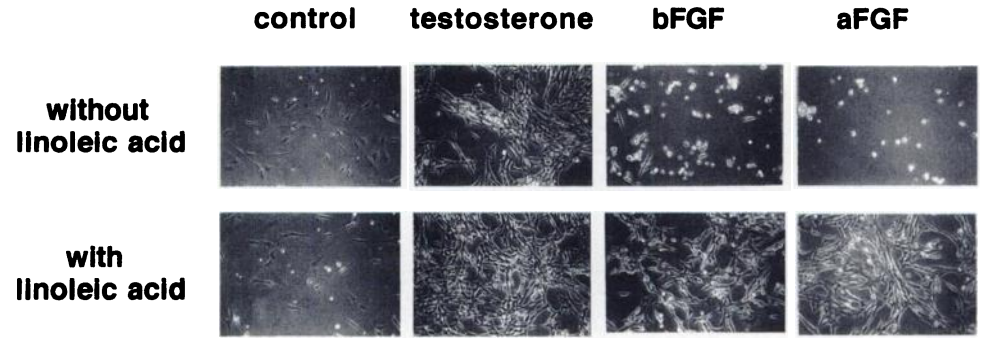

Fig. 3. Effect of LA on the cell morphology of SC-3 cells treated without or with testosterone, bFGF or aFGF. The cells were plated and treated as described in the legend of Fig. 1. The phase contrast photomicroscopy was taken on day 6.

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products have been known to regulate the cellular mitogenesis via protein kinase C- as well as Ca^{2+}-dependent pathways (29). The phospholipase A2-catalyzed products such as prostaglandin E2 (30), leukotriene C4 (31), and leukotriene D4 (32) were also demonstrated to modulate the cell growth. However, the molecular mechanism of FA-dependent regulation of the cell growth is largely obscure. LA was proposed to enhance the growth of mouse mammary tumor cells, through 5-lipoxygenase-dependent elevation of intracellular cyclic GMP (33). This pathway might not be involved in the process of LA-dependent growth enhancement of SC-3 cells, because neither cyclooxygenase inhibitor nor 5-lipoxygenase inhibitor can block the LA action. In addition, oleic acid, which is not metabolized to prostaglandins, was able to stimulate the growth of SC-3 cells. This suggests that a mechanism other than enhanced prostaglandin synthesis is involved in the FA-stimulated growth. In consistent with this consideration, the addition of arachidonic acid or linolenic acid into HMB medium resulted in the death of the SC-3 cells. These results might suggest that LA added into HMB medium cannot be metabolized into linolenic acid or arachidonic acid in a quantity sufficient for exhibiting their cytocidal effect on the SC-3 cells. Polyunsaturated FAs with 3 or 4 double bonds were observed to be cytocidal against the growth of breast and prostate cancer cells, although these previous results were obtained by using high concentrations of FA in serum-supplemented media (34–36). Accordingly, the present study indicates that these unsaturated FAs exert the growth-supporting ability without metabolic activation. Recently, polyunsaturated FAs have been reported to directly activate the peroxisome proliferator-activated receptor-retinoid X receptor heterodimers (37). The possibility should be examined in a future study that this new signal pathway of FAs is involved in the mechanism of LA-enhanced growth of the SC-3 cells.

LA is required for the continuous growth of SC-3 cells stimulated with FGF family proteins, while androgen is able to sustain the continuous growth without exogenous addition of LA. Thus, one may speculate that androgen can induce LA or LA-related metabolites in the SC-3 cells. However, LA could further enhance the growth of SC-3 cells maximally stimulated with androgen, suggesting that LA uses the signal pathway in a different manner from that induced by androgen. Actually, our gas chromatography-mass spectrometry analysis of FAs revealed that stimulation with androgen for 3 days failed to induce the biosynthesis of LA and to significantly elevate the contents of oleic acid and palmitoleic acid in total as well as free fractions of FAs extracted from SC-3 cells, when compared with the results obtained by using FGF-stimulated cells. In addition, the concentrations of these unsaturated FAs in the conditioned media were not changed in response to androgen stimuli (data not shown). These results suggest that androgen induces a LA-unrelated molecule, which can also support the continuous growth of SC-3 cells stimulated with FGF family proteins.

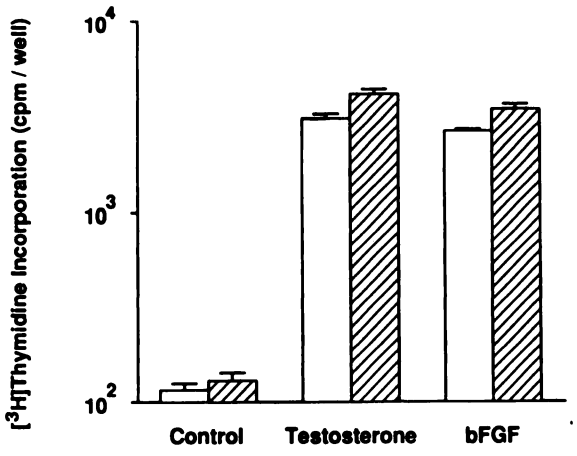

Fig. 4. Lack of the stimulatory effects of LA on the DNA synthesis of SC-3 cells. The cells were plated on triplicate wells as described in "Materials and Methods." On the following day, the cells were kept in HMB medium for 24 h to be quiescent. The cells were stimulated with 10^{-8} M testosterone or 2 ng/ml bFGF in the absence (□) or presence (▨) of 10 μg LA for 24 h. Their ability to synthesize DNA was measured. *Columns*, mean; *bars*, SE.

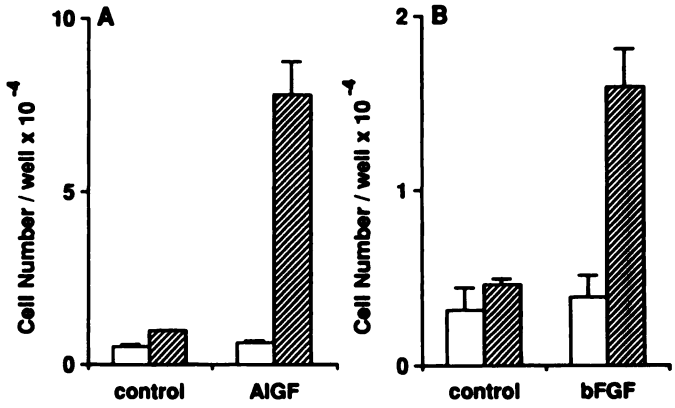

Fig. 5. Effects of LA on AIGF-stimulated SC-3 cells (*A*) and on bFGF-stimulated NIH3T3 cells (*B*). (*A*) SC-3 cells were stimulated with approximately half maximum concentration of purified AIGF in the absence (□) or presence (▨) of 10 μg/ml LA for 6 days. (*B*) NIH3T3 cells were stimulated with 2 ng/ml bFGF in the absence (□) or presence (▨) of 10 μg/ml LA for 6 days. *Columns*, mean; *bars*, SE.

Finally, we would like to discuss the present observation in relevance to clinical aspects. Androgen induces an autocrine growth factor (AIGF) in transformed target cells. With regard to activating FGF receptor, AIGF is identical to bFGF or aFGF which is ubiquitously present in a wide variety of tissues. LA or its related FAs could promote the sustained growth of the transformed cells stimulated with FGF family proteins. Even in the absence of androgen stimuli, therefore, a high concentration of LA could progress androgen-dependent cancer cells in concert with the FGF family proteins. The restriction of these mono- or diunsaturated FAs seems to be an important therapeutic target in order to prevent the progression of FGF- as well as androgen-dependent cancers.

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