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Increase in Collagen Production with Loss of Androgen Responsiveness in Cultured Androgen-responsive Shionogi Carcinoma 115 Cells

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Abstract—*The collagen production of androgen-responsive and -unresponsive Shionogi carcinoma* 115 *cells was investigated by culturing them in a medium with or without testosterone. Androgen-unresponsive cells were obtained by culturing a cloned androgen-responsive cell in a testosterone-free medium for* 12 *weeks. The collagen production of androgen-responsive cells slightly increased in the absence of testosterone, whereas testosterone did not affect the collagen production of androgen-unresponsive cells. Androgen-unresponsive cells produced* 3–4 *times more collagen than androgen-responsive cells. The major collagen produced by both androgen-responsive and -unresponsive cells migrated to the same position in sodium dodecylsulfate:polyacrylamide gel electrophoresis. The present results indicate that the collagen production of androgen-responsive Shionogi carcinoma* 115 *cells increases with the loss of androgen responsiveness in culture.*

INTRODUCTION

ANDROGEN-DEPENDENT mouse mammary carcinoma, Shionogi carcinoma 115 (SC115), was established in 1964 by Minesita and Yamaguchi [1, 2]. The original tumor occurred spontaneously as an androgen-independent adenocarcinoma of mammary origin in a female mouse. After 19 generations of passage in male DS mice, the tumor was found to be androgen-dependent; the tumor did not grow in either female or castrated male mice, but grew in these mice treated with androgen. Since SC115 cells show androgen dependence *in vitro* [3–7] as well as *in vivo* [2, 8, 9], they have been extensively used to study the mechanism of cell proliferation regulated by steroid hormones in culture.

SC115 cells cultured in the presence of androgen retain androgen responsiveness. However, when cultured without androgen for several weeks, they lose androgen responsiveness [10–13]. Androgen-responsive cells have a fibroblast-like morphology and show anchorage-independent growth. On the other hand, androgen-unresponsive cells are flattened and require attachment to a substrate for growth. Couchman *et al.* [11] have shown that the loss of androgen responsiveness is correlated with the appearance of focal adhesions associated with prominent microfilament bundles and with the production of more fibronectin. It has been shown that cultured cells produce a substrate to which they attach and on which they spread and grow, and that the substrate affects the morphology and growth of cells [14–16]. Since no study has been undertaken about changes in substrate production by SC115 cells with loss of androgen responsiveness and since collagen is one of the major components of the substrate [14–16], we investigated changes in collagen production by SC115 cells with loss of androgen responsiveness.

MATERIALS AND METHODS

Cells

A cloned androgen-responsive cell was obtained from the SC115 tumor grown in a male DS mouse as previously described [17], and maintained in a Dulbecco's modified Eagle's medium (DMEM)

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with 5% fetal calf serum (FCS) containing 0.01 μg/ml testosterone. Androgen-unresponsive cells were obtained by culturing the cloned androgen-responsive cell in DMEM with 2% FCS for 12 weeks. They were maintained in DMEM with 2% FCS.

Cell growth in monolayer culture

Androgen-responsive and -unresponsive cells were suspended in DMEM with 2% FCS, and plated at 2×10^4 cells in a 35 mm plastic tissue-culture dish. On the following day, the medium was replaced by DMEM (3 ml) with 2% FCS containing various concentrations of testosterone. The medium was changed every 3 days. Cells were harvested on the 12th day of culture, and the number of cells was counted using a hemocytometer.

Cell growth in agarose gel

One and a half milliliter aliquots of 0.5% agar in DMEM with 5% FCS were placed in 35 mm plastic dishes and allowed to solidify at room temperature. Trypsinized cells were suspended at a concentration of 670 cells/ml in 0.33% agar in DMEM with 5% FCS containing no testosterone or 3×10^{-8} M testosterone. One and a half milliliter aliquots of the cell suspension were spread over the basal layers of 0.5% agar and solidified at room temperature. The cells were cultured for 20 days and the cell colonies of minimum diameter 0.11 mm were counted.

Culture for estimation of collagen synthesis

Cells were suspended in DMEM with 2% FCS, and plated at 5×10^4 cells in a 60 mm plastic tissue culture dish. On the following day, the medium was replaced by DMEM (5 ml) with 2% FCS containing no or 0.01 μg/ml of testosterone. Two days later, the medium was again exchanged for the medium (3 ml) of the same composition except that it contained [2,3-^{3}H]proline (20 μCi/ml, specific activity 55 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.), ascorbic acid (25 μg/ml) and β-aminopropionitrile (65 μg/ml, Sigma Chemical Co., St Louis, MO, U.S.A.). After 48 h of culture, the medium was collected and the number of cells was counted.

Collagen synthesis

The synthesis of collagen was estimated using purified collagenase from *Clostridium histolytium* (Form III, Advance Biofacture Corp., Lynbrook, NY, U.S.A.) according to the method of Peterkofsky *et al.* [18]. The medium samples (0.5 ml) were precipitated with an equal volume of 20% (w/v) trichloroacetic acid (TCA) at 4°C for 1 h. The precipitates were collected by centrifugation at 1000 **g** for 10 min, washed five times with 10% (w/v) TCA, and resuspended in 0.5 ml of 0.5 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (hepes), pH 7.2. The radioactivity of 50 μl sample was determined by mixing with 10 ml of Atomlit (New England Nuclear) to estimate the synthesis of total TCA-precipitable proteins. The counting efficiency was 52.5%. A 100 μl sample was added to 110 μl of 0.5 M hepes (pH 7.2) containing 4.5 mM $CaCl_2$ and 22.7 mM *N*-ethylmaleimide (Sigma Chemical Co.). Then, one sample was incubated with 50 units of collagenase dissolved in 20 μl of 0.05 M tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 7.6) containing 5 mM $CaCl_2$ at 37°C for 6 h, while a control sample was incubated in similar conditions without addition of the enzyme. After the incubation, proteins were precipitated in 10% (w/v) TCA:0.25% (w/v) tannic acid with bovine serum albumin (100 μg), and the radioactivity in the supernatant was determined. The counting efficiency was 46.5%. The amount of collagen synthesized was calculated by measuring the amount of labeled material solubilized by collagenase and subtracting the radioactivity in the supernatant of the control sample. Corrections for a high proline content in collagen were made according to the method of Diegelmann and Peterkofsky [19].

Electrophoresis of collagen

Mouse type I collagen (50 μg) prepared from mouse tail tendon as previously described [20], was added to the medium. Collagen was extracted from the medium according to the method of DeClerk *et al.* [15] and lyophilized in small aliquots. Some lyophilized samples were dissolved in 300 μl of 0.5 M hepes (pH 7.2) and treated with 150 units of collagenase at 37°C for 16 h in the same incubation condition as described above. Collagenase-digested samples were dialyzed against 0.1 M acetic acid, frozen and lyophilized. All the lyophilized samples with or without collagenase digestion were dissolved in 1 ml of 0.1 M acetic acid containing pepsin (100 μg/ml, Sigma Chemical Co.) and incubated at 4°C for 24 h. After the incubation, the samples were frozen and lyophilized. Then they were dissolved in 0.125 M Tris–HCl (pH 6.8) containing 0.1% (w/v) sodium dodecylsulfate (SDS) 1 M urea, 20% (w/v) glycerol, 0.1% (w/v) bromophenol blue and 50 mM dithiothreitol. SDS:polyacrylamide (0.1% SDS) gel electrophoresis was performed according to the method of Laemmli [20, 21]. A 3% stacking and 5% separation gel was used, and mouse type I collagen was subjected to electrophoresis as a marker. Radiolabeled protein bands were detected by autoradiography after fluorography in Enhancer (New England Nuclear).

RESULTS

The effect of testosterone on the cell growth

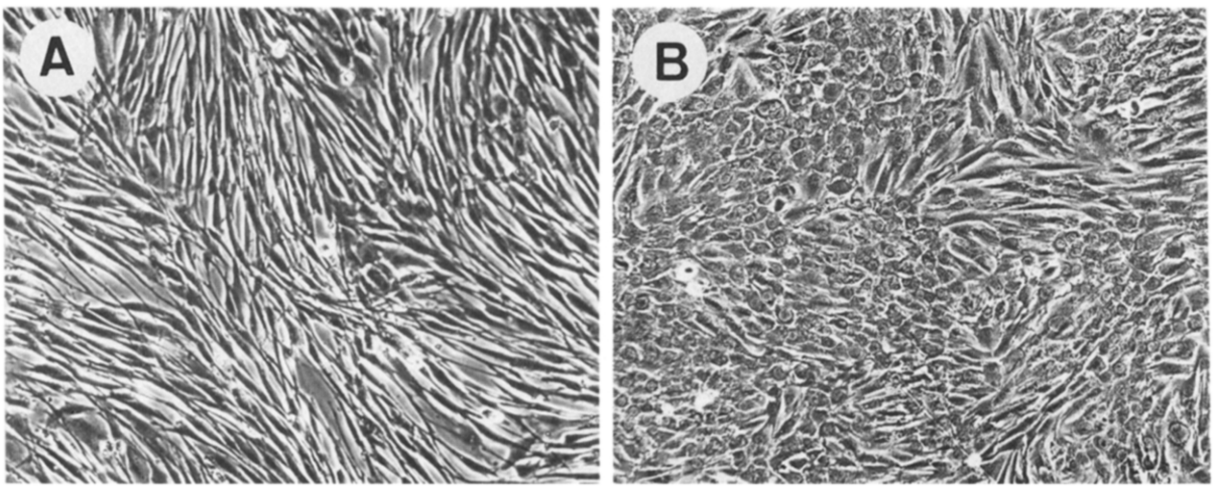

Fig. 2. Morphology of androgen-responsive and -unresponsive cells. × 100. A: androgen-responsive cells. B: androgen-unresponsive cells.

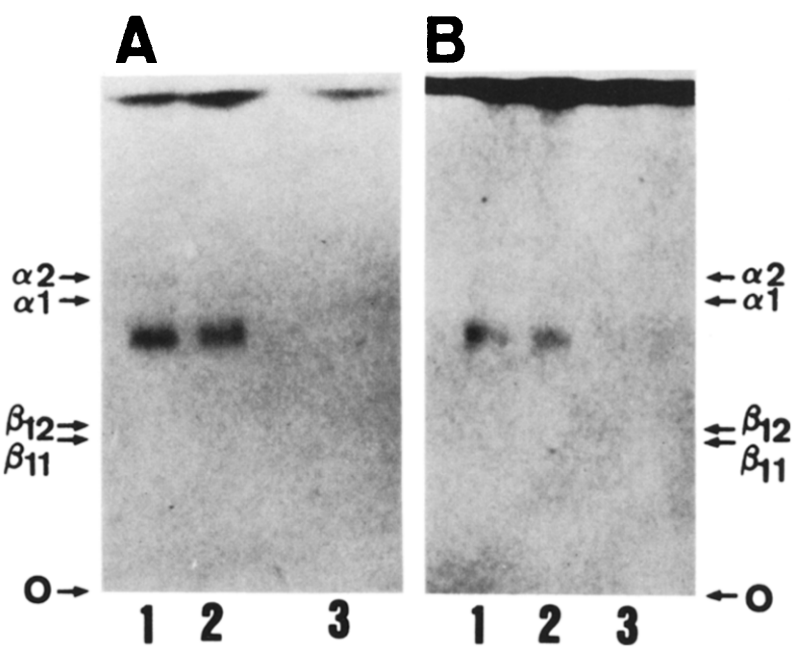

Fig. 3. SDS:polyacrylamide gel electrophoresis of the collagenous protein secreted into the medium. A: Collagenous protein secreted by androgen-unresponsive cells. B: Collagenous protein secreted by androgen-responsive cells. Lane 1: a sample obtained from the medium with testosterone. Lane 2: a sample obtained from the medium without testosterone. Lane 3: a collagenase-digested sample obtained from the medium without testosterone. O: origin, α_1,α_2, β_{12} and β_{11}: α chains and β components of mouse type I collagen.

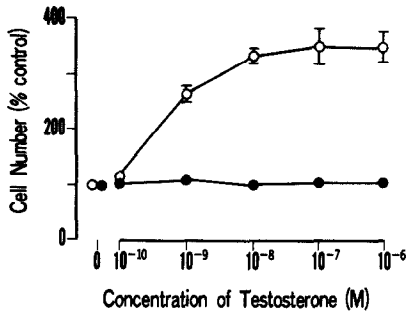

Fig. 1. Efect of testosterone on the growth of androgen-responsive and -unresponsive cells. Cells were plated at 2 × 10⁴ cells per dish. The number of cells was counted on the 12th day of culture. The numbers of androgen-responsive (○) and -unresponsive (●) cells are shown as the percentage of the mean number of cells cultured without testosterone. The mean numbers of androgen-responsive and -unresponsive cells were 39.6 and 53.3 × 10⁴, respectively. Each point represents mean ± S.E. from five dishes.

was examined in monolayer cultures (Fig. 1). The growth of androgen-responsive cells was stimulated by 10^{-9} M testosterone. The maximal growth was attained at concentrations above 10^{-8} M. The growth of androgen-unresponsive cells was not affected by testosterone regardless of its concentration. Androgen-responsive cells were elongated and had a fibroblast-like morphology, whereas androgen-unresponsive cells were flattened, having a polygonal or spindle shape (Fig. 2).

The cell growth was also investigated in soft agar. One thousand cells per dish were cultured in the presence or absence of 3×10^{-8} M testosterone. Androgen-responsive cells produced 37.0 ± 1.4 colonies (mean ± S.E., $n = 7$) in the presence of testosterone, but the number of colonies was reduced to 11.7 ± 1.0 (mean ± S.E., $n = 7$) in the absence of testosterone. On the contrary, androgen-unresponsive cells produced no colonies with or without testosterone.

The synthesis of collagen and total TCA-precipitable proteins secreted into the medium was examined in the presence and absence of testosterone (Table 1). The synthesis of collagen was assayed as bacterial collagenase-sensitive proteins. Androgen-responsive cells synthesized a significant amount of collagen in the presence of testosterone, while they produced slightly more collagen in the absence of testosterone. The relative amount of collagen was significantly higher in the absence of testosterone. The synthesis of collagen and total TCA-precipitable proteins by androgen-unresponsive cells was not affected by testosterone. Androgen-unresponsive cells produced 3–4 times more collagen than androgen-responsive cells. The relative amount of collagen produced by androgen-unresponsive cells was also significantly higher than that produced by androgen-responsive cells.

Collagenous proteins incorporated with [^{3}H]proline were separated from the medium, treated with pepsin, and analyzed by SDS:polyacrylamide gel electrophoresis and fluorography (Fig. 3). The major radioactive bands of collagenous protein produced by both androgen-responsive and -unresponsive cells with or without testosterone were detected at the same position slightly more slowly than the mouse α_1(I) chain, in electrophoresis using a reducing agent. The position of the radioactive bands was not altered in electrophoresis without a reducing agent (data not shown). The radioactive bands completely disappeared after the bacterial collagenase digestion (Fig. 3). When the collagenous protein extracted from the medium was sequentially precipitated in 50 mM Tris–HCl (pH 7.5) by 1.0, 1.7, 2.6 and 4.4 M NaCl according to the method of Hata *et al.* [22], the collagenous protein representing the major radioactive band precipitated at a NaCl concentration of 4.4 M (data not shown).

DISCUSSION

The results show that the collagen production by SC115 cells increased with the loss of androgen responsiveness. The loss of androgen responsiveness of SC115 cells has been shown to be associated with the loss of several characteristics of transformed cells [10–12]. Both androgen-responsive and -unresponsive cells are immortalized cells and induce tumor growth in nude mice [10–13]. However, unlike responsive cells, unresponsive cells show increased serum requirement for growth, contact inhibition and anchorage-dependent growth as normal cells [10–13]. We also confirmed the anchorage-dependent growth of androgen-unresponsive cells in the present study. It has been shown that normal cells produce more collagen than transformed cells [14, 16]. Thus, the increased production of collagen by androgen-unresponsive cells may reflect a certain aspect of the loss of characteristics of transformed cells.

The culture of androgen-responsive cells in the testosterone-free medium resulted in slightly more production of collagen. This may be an early change in transition from androgen-responsive cells to androgen-unresponsive cells. A similar transitional change has also been reported in microfilament and focal adhesion distribution [11].

Androgen-unresponsive cells produced more collagen than androgen-responsive cells. Furthermore, androgen-unresponsive cells have been shown to have more fibronectin in a cell-substrate contact area than androgen-responsive cells [11]. Since fibronectin binds cells to collagen [14, 16, 23], the flattened appearance of androgen-unresponsive cells may be attributed to the increased production of collagen and fibronectin together with many focal adhesions reported by Couchman *et al.* [11]. We

Table 1. Synthesis of collagen and TCA-precipitable proteins in the medium by androgen-responsive and -unresponsive cells

Cell	Presence of testosterone	No. of cultures	Collagen* (dpm/cell/48 h)	TCA-precipitable proteins* (dpm/cell/48 h)	Relative amount of collagen (%)*†
Responsive	Yes	6	2.94 ± 0.12	33.5 ± 2.1	1.8 ± 0.1
	No	6	4.12 ± 0.48	34.0 ± 4.6	2.5 ± 0.2‡
Unresponsive	Yes	5	12.77 ± 1.15§	53.3 ± 4.3§	5.5 ± 0.3§
	No	6	12.15 ± 0.95§	47.1± 4.0	6.1 ± 0.1§

Androgen-responsive and -unresponsive cells were cultured in a medium containing [^{3}H]proline (20 μCi/ml), ascorbic acid (25 μg/ml), and β-aminopropionitrile (65 μg/ml) in the presence or absence of testosterone (0.01 μg/ml) for 48 h.
*Mean ± S.E.
†The relative amount of collagen was calculated using the following formula [19]:

$$\% \text{ of collagen} = \frac{\text{collagen dpm} \times 100}{(\text{TCA-precipitable proteins dpm} - \text{collagen dpm}) \times 5.4 + \text{collagen dpm}}.$$

‡$P < 0.05$, significant difference between the values obtained with and without testosterone.
§$P < 0.05$, significant difference between the values for androgen-responsive and -unresponsive cells in the same culture conditions.

noticed that androgen-unresponsive cells were more resistant to treatment with trypsin (0.5%) and ethylenediaminetetraacetic acid (EDTA, 0.02%) than androgen-responsive cells. This agrees with the tight adhesion of androgen-unresponsive cells to a substrate.

The major collagenous protein produced by both androgen-responsive and -unresponsive cells migrated to the same position in SDS:polyacrylamide gel electrophoresis. Since the band of this protein disappeared after collagenase digestion of the samples, this protein seems to be collagen. The major collagenous protein migrated to the position slightly more slowly than the α_1(I) chain in SDS:polyacrylamide gel electrophoresis. The migration of this protein was not altered regardless of the presence or absence of a reducing agent. Thus, this collagenous protein would not be the α_1(III) chain [24]. The salt fractionation showed that this protein precipitated at neutral pH at NaCl concentrations between 2.6 and 4.4 M. This dismisses the possibility that this protein might be types I and IV collagen [24]. Sakakibara *et al.* [25] have shown that the α_1(I) trimer produced by human carcinoma cells migrated to a position slightly more slowly than the α_1(I) chain, and Moro and Smith [26] have shown that the α_1(I) trimer precipitated at neutral pH at NaCl concentrations between 3.4 and 4.2 M. Furthermore, several reports have shown the production of the α_1(I) trimer by tumor cells *in vitro* [15, 25, 27]. Thus, the major collagenous protein produced by both androgen-responsive and -unresponsive SC115 cells might be the α_1(I) trimer.

The removal of androgen from the culture medium results in constant production of androgen-unresponsive cells from androgen-responsive cells [10–13]. The loss of androgen responsiveness is associated with the loss of several characteristics of transformed cells [10–13]. Thus, analysis of differences between these cells could be useful to reveal the mechanism of steroid-induced transformation.

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