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2 March 1981

Growth Arrest and Morphological Change of Human Breast Cancer Cells by Dibutyryl Cyclic AMP and L-Arginine

Abstract. *The growth in vitro of human breast cancer cells, line MCF-7, was inhibited by a daily supplement of* L*-arginine (1 milligram per milliliter). Arginine acted synergistically with dibutyryl adenosine 3′,5′-monophosphate (cyclic AMP) (10^{-6} molar) to enhance the growth inhibitory effect: the cell replication ceased completely within 2 days after treatment. The growth arrest accompanied a change in cell morphology and was preceded by increases in the cellular concentration of cyclic AMP, adenylate cyclase, and type II cyclic AMP–dependent protein kinase activities as well as a decrease of estrogen binding activity. The results suggest that growth of human breast cancer cells is subject to cyclic AMP–mediated regulation and that arginine may play a specific role in this process.*

Human mammary carcinomas often regress after endocrine therapy. The regression has been linked to the presence of an estrogen receptor in the tumor (*1*) and, indeed, measurement of the receptor is now used extensively as an assay to identify patients likely to respond to endocrine therapy. However, about one-half of human mammary carcinomas containing estrogen receptor do not regress after ovariectomy (*2*). It is conceivable, therefore, that estrogen receptor may interact with other cellular regulators in the growth control of mammary tumors.

Studies from our laboratory suggested that regulation of the growth of hormone-dependent mammary tumors may depend on the antagonistic action between estrogen and adenosine 3′,5′-monophosphate (cyclic AMP). Cyclic AMP arrests whereas estrogen stimulates the growth of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary tumors (*3*). During growth arrest of the tumors after either hormone removal (ovariectomy) or treatment of the hosts with N^6,$O^{2'}$-dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP), estrogen binding decreases whereas cyclic AMP binding and cyclic AMP–dependent protein kinase activity increase in the cytosol and nuclei of the tumors (*4*). Moreover, our recent studies (*5*) have shown that the growth inhibitory effect of dibutyryl cyclic AMP is enhanced by L-arginine which stimulates nicotinamide adenine dinucleotide (NAD)–dependent adenosine diphosphate ribosylation and adenylate cyclase activation in the hormone-dependent mammary tumors in vivo.

In the work reported here we explored (i) the role of cyclic AMP in the growth of human mammary cancer cells in vitro; (ii) the effect of arginine in the cyclic AMP system; and (iii) the synergistic action between arginine and dibutyryl cyclic AMP in the growth control.

We chose a human breast cancer cell line, MCF-7, as an experimental model. The MCF-7 cell line was derived from a hormone-dependent metastatic breast cancer (*6*). Although the growth of these cells does not apparently depend on exogenous estrogen, estrogen receptor is present (*7*) and antiestrogen (tamoxifen) strongly inhibits growth (*8*). Thus, MCF-7 cells appear to possess characteristics resembling those of hormone-dependent mammary tumors in vivo.

Daily supplements of L-arginine to MCF-7 cells in culture produced consistent growth inhibition. The growth inhibition was concentration dependent and was apparent within a few days. At a concentration of 1 mg of L-arginine hydrochloride per milliliter, the cell num-

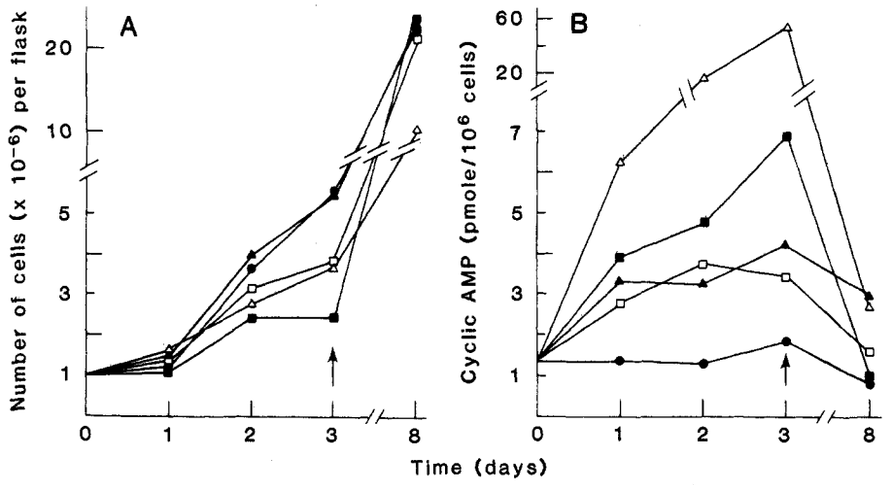

Fig. 1. Effect of L-arginine and dibutyryl cyclic AMP on the growth and cyclic AMP content of MCF-7 cells in culture. Symbols: ●, control; □, L-arginine hydrochloride (1 mg/ml); ▲, dibutyryl cyclic AMP (10^{-6} *M*); △, dibutyryl cyclic AMP (10^{-4} *M*); ■, dibutyryl cyclic AMP (10^{-6} *M*) plus L-arginine hydrochloride (1 mg/ml). The MCF-7 cells were obtained from Mason Research Institute (Rockville, Maryland) 1 day after seeding (1.0×10^6 cells per T_{75} Falcon flask). Cells were grown in the absence or presence of the additives in McCoy's 5A medium supplemented with bovine insulin (10 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and fetal calf serum (10 percent); the medium was changed every 48 hours. The additives were provided daily starting at day 1 after seeding (0 time). The arrow indicates removal of the additives. The triplicate cell counts for each experimental point never varied by more than 15 percent. Cyclic AMP was measured by the radioimmunoassay according to the acetylation procedure (*22*) exactly as described (Collaborative Research Inc., Waltham, Massachusetts). Each point represents an average of closely agreeing duplicate determinations. The data represent one of several similar experiments that gave essentially the same results.

0036-8075/81/1002-0077$01.00/0

Table 1. Effect of dibutyryl cyclic AMP and arginine on estrogen binding and protein kinase activities in MCF-7 cells. Cytosols (the supernatant obtained by centrifugation at 105,000*g*) and nuclear extracts were prepared from the control (no treatment) and treated (daily supplements of $10^{-6}M$ dibutyryl cyclic AMP plus arginine hydrochloride, 1 mg/ml, for 3 days) MCF-7 cells as described (*16*). The cytosols and nuclear extracts were then subjected to DEAE-cellulose column chromatography as described (*17*). Protein kinase activity of the eluent from the DEAE-cellulose column was measured in the absence and presence of 1 μM cyclic AMP as described (*18*) and peak I (type I kinase) and peak II (type II kinase) activities were identified. Estrogen binding [expressed as specific binding (*19*)] was measured by the modification (*19*) of the charcoal adsorption assay of Korenman (*20*). The data represent an average of closely agreeing duplicate determinations and one of several similar experiments that gave essentially the same results. Protein concentrations were measured by the method of Lowry *et al.* (*21*). The plus and minus signs indicate, respectively, the presence or absence of cyclic AMP in the assay mixture.

Treatment	Estrogen binding activity (fmole/mg protein)		Protein kinase activity (pmole/5 min-mg protein)							
			Cytosol				Nuclear extract			
			Type I		Type II		Type I		Type II	
	Cytosol	Nuclear extract	−	+	−	+	−	+	−	+
None	90	250	105	270	20	50	10	15	12	15
Dibutyryl cyclic AMP plus arginine	65	127	145	170	45	120	12	15	30	60

ber decreased to 60 percent of that of untreated cells by day 3 (Fig. 1A). Dibutyryl cyclic AMP at $10^{-4}M$ produced growth inhibition similar to that produced by arginine, but at $10^{-6}M$ had no inhibitory effect (Fig. 1A). When dibutyryl cyclic AMP at $10^{-6}M$ was added along with arginine (1 mg/ml), however, the growth inhibition was greatly enhanced: the increase in cell number ceased within 2 days (Fig. 1A) and the growth arrest was maintained for up to 10 days as long as the treatment was continued.

The synergistic growth inhibitory effect of dibutyryl cyclic AMP and arginine was also observed when arginine methyl ester, an analog of arginine, was substituted at a concentration of 50 μg/ml. However, when other amino acids, such as lysine (1 mg/ml) or tryptophan (50 μg/ml), were combined with dibutyryl cyclic AMP, growth inhibition was not enhanced, although a 20 percent decrease in the cell growth rate was observed with tryptophan alone (data not shown).

Viability of cells that were treated with arginine and dibutyryl cyclic AMP was indicated by their exclusion of trypan blue (data not shown) and their ability to resume growth after removal of additives (Fig. 1A). Moreover, the number of cells that were released into the medium during the 72 hours of culture was negligible in both treated and untreated cells. Thus growth inhibition of MCF-7 cells by arginine and dibutyryl cyclic AMP appears to be due to a decrease in the rate of cell replication without affecting cell viability.

The growth inhibition by arginine and dibutyryl cyclic AMP was preceded by an increase in the cellular concentration of cyclic AMP. Within 1 day after either arginine (1 mg/ml) or dibutyryl cyclic AMP ($10^{-6}M$) treatment, the cyclic AMP level increased and remained high (two times higher than the control value) (Fig. 1B). Arginine plus dibutyryl cyclic AMP treatment resulted in a higher increase in the concentration of cyclic AMP; the concentration increased by more than three and five times over the control values at days 1 and 3, respectively (Fig. 1B). The highest increase in cyclic AMP occurred in cultures treated with $10^{-4}M$ dibutyryl cyclic AMP; the increase was 30-fold at day 3 (Fig. 1B). The concentrations of cyclic AMP decreased in the cells that resumed growth after removal of the supplements (Fig. 1).

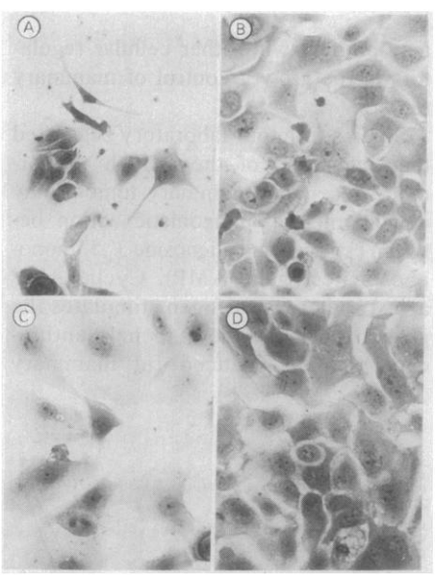

Fig. 2. Effect of L-arginine and dibutyryl cyclic AMP on the morphology of MCF-7 cells. (A and B) The cells were grown in regular medium (see legend to Fig. 1) for (A) 1 day and (B) 5 days. (C and D) The cells were grown for 5 days in regular medium plus L-arginine hydrochloride (1 mg/ml); then the cells in (D) were grown in regular medium only for another 3 days. The cells were seeded at a density of 1×10^6 cells per flask and the medium was changed every 48 hours. The photograph was taken after the cells were stained with hematoxylin and eosin (×290). The morphology of cells treated with either $10^{-4}M$ dibutyryl cyclic AMP or $10^{-6}M$ dibutyryl cyclic AMP plus L-arginine hydrochloride (1 mg/ml) was similar to that of cells shown in (C).

Figure 1 shows that both arginine and dibutyryl cyclic AMP caused an increase in cellular cyclic AMP, and that this increase was correlated with the inhibition of cell growth. However, the maximum growth inhibition, that is, arrest of cell replication, occurred when the concentration of cyclic AMP increased moderately (arginine plus dibutyryl cyclic AMP treatment) rather than excessively ($10^{-4}M$ dibutyryl cyclic AMP treatment). The results support the hypothesis (*9*) derived from studies of mammary tumor in vivo that arrest of cell growth may require an optimum concentration of cellular cyclic AMP and that both sub- and supraoptimum concentrations of cyclic AMP may stimulate rather than inhibit growth.

The increase in the concentration of cyclic AMP in MCF-7 cells upon the addition of arginine followed a sharp increase in adenylate cyclase activity: The basal and fluoride-stimulated activities (*10*) increased by three and two times, respectively, within 3 hours after treatment (data not shown). A twofold increase in the activity of the cyclic AMP–phosphodiesterase with low Michaelis constant (*11*) also occurred within 1 day after supplementation with arginine (data not shown). The activities of adenylate cyclase and phosphodiesterase decreased in the cells that resumed growth after removal of the supplement.

We have shown previously (*4*) that cyclic AMP–dependent protein kinase activity is inversely related to estrogen-binding activity in hormone-dependent

mammary tumors in vivo during their growth and regression after hormone withdrawal or treatment with dibutyryl cyclic AMP. Table 1 shows a similar inverse relation between cyclic AMP–dependent protein kinase type II and estrogen binding in MCF-7 cells. By DEAE-cellulose column chromatography we found that the cytosols from growing MCF-7 cells exhibited two major peaks of protein kinase activity that was stimulated by cyclic AMP: one eluted at low ionic strength [type I enzyme (*12*)] and the other eluted at high ionic strength [type II enzyme (*13*)]. Both types I and II kinase activities were also detected in MCF-7 cell nuclei but in greatly reduced amounts. At 3 days after the treatment of cells with dibutyryl cyclic AMP plus arginine, type II protein kinase activity increased twofold in the cytosol whereas type I enzyme activity decreased by 40 percent (Table 1). Moreover, type II enzyme activity showed a fourfold increase in the nuclei of the growth-arrested cells, whereas type I enzyme activity did not change (Table 1). Table 1 also shows that in the growth-arrested cells estrogen binding activity decreased by 30 percent and 50 percent in the cytosol and nuclei, respectively. Thus the decreases in both estrogen binding and type I cyclic AMP–dependent protein kinase activities were inversely related with the increase of type II protein kinase activity in the growth-arrested MCF-7 cells. The results suggest that estrogen receptor and type I cyclic AMP–dependent protein kinase may serve as a positive effector for cell growth, whereas type II protein kinase serves as a negative signal.

The growth arrest of MCF-7 cells by arginine and dibutyryl cyclic AMP was accompanied by a striking change in cell morphology. As shown in Fig. 2, the cytoplasm of the treated cells was greatly enlarged without appreciable change in the size of the nuclei (compare C with B). Upon removal of the supplement the cell morphology returned to that of untreated cells and the cell number increased (Fig. 2D). Cyclic AMP–induced inhibition of cell proliferation is often associated with a change in cell morphology, synthesis of specialized cell products and cell differentiation (*14*). Since both arginine-induced and dibutyryl cyclic AMP–induced growth inhibition of MCF-7 cells was preceded by an increase in cellular cyclic AMP, the morphological change may be specifically associated with the increase in cyclic AMP.

The results of this study in vitro confirms our previous finding in vivo (*15*) that dibutyryl cyclic AMP and arginine inhibit the growth of mammary tumors. Our results, therefore, suggest that dibutyryl cyclic AMP and arginine may have therapeutic potential for breast cancer in humans.

YOON SANG CHO-CHUNG
TIMOTHY CLAIR
JEFFREY S. BODWIN
BELA BERGHOFFER

Cellular Biochemistry Section, Laboratory of Pathophysiology, National Cancer Institute, Bethesda, Maryland 20205

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1 May 1981; revised 3 August 1981

Skeletal Muscle: Length-Dependent Effects of Potentiating Agents

Abstract. *The ability of vertebrate skeletal muscle to contract more vigorously than normal in the presence of potentiating agents depends on the initial length of a muscle cell. Other factors such as the intracellular calcium ion transient, temperature, chemical nature of the potentiating agent, and the ratio of intrinsic twitch to tetanic force influence the degree of contractile potentiation but cannot account for the length dependence. At least part of a muscle cell seems normally less than fully active during contractions not only at short lengths but also at optimal sarcomere lengths.*

The sliding filament theory of muscle contraction adequately accounts for the progressive decline in force development when filament overlap is reduced by stretch (*1*). But when muscle is allowed to shorten below the sarcomere length at which filament overlap is optimal, there is a progressive decline in contractility that may be due to several factors (*1, 2*). A factor suggested several years ago is that the degree of activation may be progressively reduced as a muscle shortens (*3*). Activation in vertebrate skeletal muscle is known to be caused by a transient increase in intracellular free Ca^{2+}, and we sought to monitor length-dependent changes in free calcium transients and further study their influence on the contractility of complete muscle cells (*4*). If the intracellular Ca^{2+} concentration in cells with a whole assemblage of parts could be reversibly induced to approach or exceed the amount required to saturate calcium binding sites, length-dependent variations in Ca^{2+} transients should become less influential.

Live, intact cells were isolated from the tibialis anterior, semitendinosus, and iliofibularis of the frog *Rana temporaria* and studied before and after exposure to several chemical agents known to potentiate twitches (*5*). Striation spacings were measured optically before contraction as described (*2*). The cells were also microinjected with the Ca^{2+}-sensitive luminescent protein aequorin (*4*) to allow a correlation among intracellular Ca^{2+} transients, force of contraction, and some parameters (for example, temperature, ratio of initial twitch to tetanus force) previously shown to influence the degree of potentiation in whole muscle (*5–7*). The most reproducible results are obtained when contractions are preceded by a long period of rest. Aequorin responses and tetanic force are reduced when muscles are stimulated frequently, although twitch force is potentiated

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