Cytotactin binding: Inhibition of stimulated proliferation and intracellular alkalinization in fibroblasts

(morphogenesis/growth control/second messengers/extracellular matrix)

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ABSTRACT Cytotactin is an extracellular matrix protein that is dynamically and transiently expressed in a place-dependent fashion during development by glial cells, fibroblasts, and several other cell types. In the present study, the effects of cytotactin on cell proliferation were examined in fibroblastic cells in culture. NIH 3T3 mouse cells plated on tissue culture substrata in the presence of soluble cytotactin remained rounded for longer periods than untreated control cells, similar to their response to cytotactin-coated substrates. These rounding effects could be prevented by pretreatment of the cells with nocodazole, a microtubule-disrupting agent. Cytotactin inhibited the proliferation of fibroblasts in culture in a dose- and time-dependent manner, and this inhibition occurred even after nocodazole treatment. In addition, the presence of cytotactin inhibited proliferation stimulated by growth factors or tumor promoter. These effects on cell growth were accompanied by an early inhibition of the intracellular alkalinization that normally occurs upon mitogenic stimulation by a number of growth-promoting agents. Together these observations suggest that cytotactin is an endogenous cell surface modulatory protein and provide a possible mechanism whereby cytotactin may contribute to pattern formation during development, regeneration, tumorigenesis, and wound healing.

The alteration of cell surface protein mobility and of the state of the cellular cytoskeleton, correlated with the inhibition of cell proliferation, has been termed cell surface modulation (1). The molecular events that mediate these processes have just begun to be elucidated despite a large body of data both on second-messenger systems activated by mitogens and on molecular linkages between cell surface and cytoskeletal proteins. The original phenomena revealing cell surface modulation were observed by studying the effects on lymphocytes of the mitogen concanavalin A (Con A). Lymphocytes exhibit a biphasic response to Con A: at low concentrations, Con A is mitogenic, but at higher concentrations, the molecule inhibits proliferation. These phenomena are related to the effects of Con A on the mobility of cell surface receptors, and this is in turn constrained by interactions among microtubular components of the cytoskeleton (reviewed in ref. 1). These studies were later extended to fibroblastic and other cell types in culture (2–5).

Con A is not a physiological ligand endogenous to animal cells and therefore these observations prompted a search for endogenous ligands that might exert cell surface modulatory effects, especially during embryonic development. The present report describes cellular responses to an extracellular matrix protein, cytotactin, that suggest that it is a natural ligand capable of inducing some of the phenomena characteristic of surface modulation.

Cytotactin [also known as myotendinous antigen, tenascin, J1_{220/200}, or hexabrachion (reviewed in ref. 6)] is an extracellular glycoprotein that is synthesized by glia of the central and peripheral nervous systems and by a variety of other cells in the developing embryo. During embryonic and neural development, it shows a highly transient and site-restricted distribution (7, 8). In addition to being critical to cell movement in embryogenesis, it has been shown to be dynamically and differentially expressed in a variety of tumors, as well as during regeneration and wound healing (reviewed in ref. 6). *In vitro* studies suggest that the molecule has profound effects on cell morphology. For example, cells cultured on a cytotactin substratum fail to flatten, remaining rounded for prolonged periods of time even though they are alive and healthy (9–15). The presence of cytotactin slows cell migration *in vitro* (9, 11, 12) and *in vivo* (15) and, in developing neurons, it can cause growth-cone collapse on otherwise permissive substrates (16–18). These effects can provide powerful constraints on a variety of developmental events.

A rounded cell state generated by any number of means is correlated with a decrease in cell proliferation (19) and, at least in some cases, this effect is due to an inhibition of the intracellular alkalinization that appears to be required for cell cycle progression (reviewed in refs. 20 and 21). Alkalinization is due to the action of the Na⁺/H⁺ antiporter, the phosphorylation of which is correlated with growth factor-stimulated intracellular pH (pH_i) increase (22). The present studies were focused mainly on the effects of cytotactin on the proliferation of fibroblastic cells in culture as well as on the concomitant effects on pH_i. Cultured cells plated in the presence of cytotactin exhibited a markedly lower incorporation of [³H]thymidine than did their untreated counterparts. Growth factor-stimulated [³H]thymidine incorporation was also inhibited in the presence of cytotactin. Studies of the time course of the cytotactin effects suggested that they last 30–40 hr, after which the cells begin again to synthesize DNA. In conjunction with these striking effects on cell proliferation, under certain conditions cytotactin was found to inhibit the intracellular alkalinization that is generally associated with induction of the proliferative state.

MATERIALS AND METHODS

Preparation of Cytotactin. Cytotactin was prepared from embryonic day 14 chick brains as previously described (23), with the following modifications. The supernatant from the phosphate-buffered saline (PBS) extract was brought to 8% (wt/vol) sucrose, ammonium sulfate was added to 30% saturation, and the resultant suspension was subjected to low-speed centrifugation (Sorvall GSA rotor, 15,000 rpm). The 30% supernatant was adjusted to 50% ammonium sulfate; the resulting pellet was the starting material for the affinity purification and gel filtration as described (23). In the

final preparation, the cytotactin-binding proteoglycan (23, 24) was undetectable.

Cell Culture. NIH 3T3 cells (American Type Culture Collection CRL 1658), used for all experiments, were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 1 mM glutamine and 10% calf serum in an atmosphere of 10% CO₂ in air. The cells were made quiescent by growing at high density for at least 2 days in the same medium but including only 0.5% calf serum (medium A). In some experiments, quiescence was achieved by allowing the cells to remain at a confluent density without medium change for 5 days. Cells for thymidine incorporation experiments were prepared by the low trypsin plus calcium (LTC) method (25).

Thymidine Incorporation and Normalization. Thymidine incorporation was measured as described (26). Briefly, the cells in medium A were pulse-labeled with [³H]thymidine (20 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) at 10 μ Ci/ml for 2 hr from 20 to 22 hr after treatment with growth factors or cytotactin except as noted otherwise in the time-course studies.

Two methods of analysis were used to account for possible cell loss due to poorer binding to the substratum in the presence of cytotactin: (i) parallel cultures were labeled with [³H]thymidine prior to the experiment and then treated identically to their pulse-labeled counterparts; (ii) cells were labeled intrinsically with [³⁵S]methionine prior to the experiment and then the LTC cultures prepared from these cells were labeled with [³H]thymidine. Both methods yielded similar results but the latter method was used more routinely since it could be performed in a single culture. At the highest concentrations of cytotactin the ³⁵S label was $\approx$ 70% of that in untreated cultures. The windows for ³H and ³⁵S counting were selected for minimum overlap; no ³H counts were detected in the ³⁵S channel, and 40% of the total counts in the ³H channel resulted from spillover from the ³⁵S channel; therefore, adjusted cpm values were calculated using the formula (³H - 0.4³⁵S)/³⁵S. By these means it was possible to express the results of the thymidine incorporation in terms of a ratio to an internal label related to the actual cell number initially present at the start of the experiment.

The majority of experiments were performed in 96-well tissue culture dishes (Falcon 3072). Soluble cytotactin was added at the time of plating except where indicated. For growth factor additions, LTC preparations from quiescent cells were incubated with cytotactin and the indicated growth factor at the time of plating; in all cases, the concentration of growth factor was maximal for the stimulation of thymidine incorporation in 3T3 cells.

Measurement of pH_i. pH_i was measured using the fluorescent dye 2',7'-bis-(2-carboxyethyl-5-(and 6)-carboxyfluorescein (BCECF, acetoxymethyl ester from Molecular Probes) and LTC cells (5×10^5 per ml) plated in 0.25 ml of medium A on rectangles (1 $\times$ 2 cm) cut from Falcon 3013 tissue culture flasks. Fluorescence was measured using an SPF-500C spectrofluorometer (SLM Aminco, Urbana, IL) with a band pass of 2.5 nm (excitation) and 5 nm (emission). Cells were labeled with 10 μ M BCECF acetoxymethyl ester for 10 min in medium B plus 3.7% NaHCO₃ (medium B was 119 mM NaCl/5 mM KCl/25 mM Pipes, 5.6 mM glucose/1 mM CaCl₂/0.4 mM MgCl₂/0.1% bovine serum albumin, pH 7.2), rinsed twice in medium A preequilibrated with CO₂, and allowed to incubate an additional 10 min in the second medium A change. Fluorescence measurements were made in CO₂-equilibrated medium B at excitation wavelengths of 505 nm and 439 nm with emission at 535 nm; subsequently the values of the medium alone were determined and subtracted from the total to account for dye leakage. pH_i is proportional to the ratio of the fluorescence intensity at an excitation wavelength of 505 nm to that at 439 nm. A pH standard curve

FIG. 1. 3T3 fibroblasts were plated on 24-well plastic tissue culture plates (Falcon 3047) in medium with 0.5% calf serum with (A) or without (B) cytotactin (7.5 μ g/ml). Photographs were taken 60 min after plating. (Bar = 10 μ m.)

was prepared using buffers of known pH and cells treated with the ionophore nigericin (27).

RESULTS

Maintenance of Cell Rounding by Exposure of Fibroblasts to Cytotactin. When fibroblasts were plated on tissue culture dishes in the presence of cytotactin, the majority of cells remained rounded for up to 2 hr (Fig. 1A). This was in contrast to cells plated on tissue culture plastic alone (Fig. 1B) or cells plated in the presence of soluble fibronectin (not shown), which, in both cases, rapidly flattened within 30 min. Cells that were already flattened on the culture substratum, however, were not significantly altered in their morphology when cytotactin was added to the culture medium. Cells flattened even more rapidly than controls when pretreated with nocodazole, and the presence of cytotactin did not affect this more rapid flattening (data not shown). This result suggests that the effects of cytotactin on cell shape depend upon microtubular states.

Effects of Cytotactin on Levels of Thymidine Incorporation. 3T3 fibroblasts plated at low density exhibited high levels of proliferation as indicated by multiple rounds of thymidine incorporation with a doubling time of $\approx$ 17 hr (data not shown). At low density, cells rapidly incorporated [³H]thymidine even when serum was reduced from 10% to 0.5% of the culture medium. Thus during the time course of the experiments, NIH 3T3 cells plated at low density were not quiescent even when serum-depleted. When cytotactin was included at the time of plating in the low-serum incubation medium, however, the incorporation of [³H]thymidine was significantly reduced (Fig. 2). The effects of cytotactin were dose-dependent, with half-maximal inhibition at a cytotactin

FIG. 2. Cell proliferation of 3T3 fibroblasts and effects of cytotactin. LTC cells were plated with the indicated concentration of cytotactin (CT). Levels of thymidine incorporation are expressed as percent of untreated controls.

Table 1. Effects of short exposure to cytotactin (CT) on thymidine incorporation

	$^3\text{H}/^{35}\text{S}$ ratio	% control
PBS control	73 ± 8	100
CT (continuous)	12 ± 5	16
PBS, medium change	66 ± 6	90
CT (3 hr), medium change	17 ± 3	23

Cells were prepared as described in *Materials and Methods* and treated with cytotactin at the time of replating. After 3 hr, medium was removed and replaced with fresh medium or remained unchanged in control cultures. Cultures were pulse-labeled with [^3H]thymidine for 2 hr from 20 to 22 hr after the initial addition of cytotactin. Values represent mean $\pm$ SD of triplicate cultures.

concentration of 1–2 $\mu\text{g}/\text{ml}$. Although cytotactin was able to inhibit thymidine incorporation at higher levels of serum in the medium (up to 5%), the inhibition was never complete at these serum levels. Similar results were observed for primary chicken fibroblasts and glia and the human glioma HS683.

The effects of cytotactin were not restricted to rounded cells after LTC treatment. Cells in monolayer at subconfluent densities were also sensitive to inhibition of thymidine incorporation by cytotactin, and the inhibitory effects of cytotactin were dependent on cell density. Cells initially plated at 2×10^3 per well in 96-well plates (7×10^3 cells per cm^2) and serum-starved for 2 days after plating were inhibited 93% in the presence of cytotactin at 12 $\mu\text{g}/\text{ml}$, whereas cells plated at 2×10^4 per well (7×10^4 cells per cm^2) were inhibited only 66% (data not shown).

When cells were pretreated with nocodazole and then plated in the presence of cytotactin, thymidine incorporation was still inhibited, despite the fact that the cells flattened immediately after plating (see above). This suggests that the early effects of cytotactin on inhibition of cell proliferation are not strictly correlated with its effects on cell shape.

Time Course and Reversibility of Cytotactin Effects. Time-course studies confirmed that the effects of cytotactin oc-

curred early (<3 hr) in the incubation period. As shown in Table 1, cells were either continuously treated with cytotactin (4 $\mu\text{g}/\text{ml}$) or treated with cytotactin for 3 hr, after which the cytotactin was washed out and the medium was replaced with fresh medium. In the former case, cytotactin inhibited thymidine incorporation to 16% of that seen in controls. After washout of cytotactin, thymidine incorporation was still inhibited to 26% of control levels, suggesting that the inhibition of thymidine incorporation by cytotactin was most significant in the early part of the culture period.

Cells were able to undergo a subsequent round of division after the initial inhibition by cytotactin (Fig. 3), indicating that the presence of the exogenous protein was not toxic or otherwise harmful. When cells were treated with cytotactin and with [^3H]thymidine at the beginning of the culture period and harvested at various times (Fig. 3A) up to 48 hr, the cytotactin-treated cells appeared to be inhibited up to 40 hr of incubation. Between 42 and 48 hr, however, when the control cells had entered into a new S phase, the cytotactin-treated cells also began to synthesize DNA at a level comparable to controls (Fig. 3B). The accumulated data indicate that cytotactin has its effects early in the cell cycle and that the effects are reversible, possibly by the depletion or degradation of cytotactin or by the cells becoming refractory to its effects.

Inhibition of Growth Factor-Stimulated DNA Synthesis by Cytotactin. Cells were incubated with a variety of growth factors including epidermal growth factor, platelet-derived growth factor, acidic fibroblast growth factor, and basic fibroblast growth factor. Cells exposed to each showed an increased incorporation of [^3H]thymidine over untreated controls (Table 2). When cytotactin was included in the incubation medium at the time of growth factor addition, however, the increase in DNA synthesis was inhibited to varying extents; in every case, higher concentrations of cytotactin blocked almost completely. Similarly, PMA-stimulated DNA synthesis was blocked in the presence of

FIG. 3. Time course of cytotactin effects. (A) Cells were plated with (○) or without (●) cytotactin (15 $\mu\text{g}/\text{ml}$) together with [^3H]thymidine (10 $\mu\text{Ci}/\text{ml}$) and harvested at the indicated time (abscissa). The ordinate represents the $^3\text{H}/^{35}\text{S}$ ratio determined as described in *Materials and Methods*. Standard deviations of triplicate measurements are shown by error bars; in some cases, the bars are obscured by the circle. (B) Data from A together with that of a parallel experiment with additional time points. Ordinate indicates cytotactin-treated (+CT) cells as a percentage of control cells (–CT).

Table 2. Effects of cytotactin (CT) on growth factor-stimulated DNA synthesis

Factor*	CT, $\mu\text{g}/\text{ml}$	$^3\text{H}/^{35}\text{S}$ ratio	% control	% growth factor-treated
Exp. 1				
None	0	16.1 ± 1.4	100	
	3	4.1 ± 0.2	25	
	1.5	9 ± 2	57	
1% serum	0	58 ± 3	360	100
	3	38 ± 5	236	66
aFGF (50 ng/ml)	0	71 ± 10	441	100
	3	40 ± 1	248	56
bFGF (100 ng/ml)	0	89 ± 8	553	100
	3	26 ± 2	161	29
PDGF (25 ng/ml)	0	94 ± 13	584	100
	3	44 ± 13	273	47
Exp. 2				
None	0	41 ± 4	100	
EGF (100 ng/ml)	0	107 ± 14	261	100
	7.5	35 ± 4	85	33
PMA (1 μM)	0	126 ± 15	307	100
	7.5	36 ± 1	88	29

$^3\text{H}/^{35}\text{S}$ ratios are means $\pm$ SD of triplicate cultures. The fourth column represents each ratio value as a percentage relative to untreated control cultures; the fifth column represents the $^3\text{H}/^{35}\text{S}$ ratio of cytotactin-treated samples relative to each growth factor treatment.

*Serum, calf serum; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PMA, the tumor promoter phorbol 12-myristate 13-acetate.

Table 3. Short-term effects of cytotactin (CT) on pH_i of fibroblasts in culture

	Treatment	pH_i	ΔpH_i
Exp. 1	None, 4 hr	7.43 ± 0.01	
	CT (6 $\mu g/ml$), 4 hr	7.17 ± 0.06	0.26 ± 0.07
Exp. 2	None, 1 hr	7.32 ± 0.05	
	CT (6 $\mu g/ml$), 1 hr	7.15 ± 0.05	0.17 ± 0.10

pH_i measurements were made and pH was calculated as described in *Materials and Methods*. Values represent mean $\pm$ SD of triplicate cultures.

cytotactin (Table 2, Exp. 2). All of these results are consistent with the conclusion that cytotactin binding interferes with growth factor-stimulated cell division.

Effects of Cytotactin on pH_i . pH_i was measured in cells in the presence or absence of cytotactin (Table 3). Cells in single-cell suspension, prepared by the LTC method (25), had a pH_i between 6.8 and 7.0, a level that was somewhat variable from cell preparation to preparation. The pH_i relative to untreated controls was monitored from 1 to 4 hr after plating. Over this time, cytotactin-treated cells were more loosely attached than control cells. Over a 4-hr period, control cell pH_i rose to 7.43 ± 0.01 , whereas cells in the presence of cytotactin maintained a lower pH_i , 7.17 ± 0.06 . In subsequent experiments, the pH_i in cells exposed to cytotactin was 0.15–0.3 unit lower than that of control cells, regardless of the initial pH_i of the control cells. By 10 hr, the pH_i values of treated and untreated cells were identical (data not shown).

DISCUSSION

The present results may be summarized as follows. (i) Cells plated on tissue culture plastic in the presence of cytotactin remained rounded for prolonged periods of time. Within 2 hr, however, they flattened and were morphologically indistinguishable from control cells. The inhibition of flattening could be obliterated by pretreatment with nocodazole, a microtubule-dissociating agent. (ii) The presence of cytotactin inhibited the synthesis of DNA in fibroblasts in culture. These effects of cytotactin were dose- and time-dependent. (iii) Thymidine incorporation stimulated by growth factors or tumor promoter was also inhibited by cytotactin. (iv) The intracellular alkalization that normally accompanies growth stimulation was inhibited in the presence of cytotactin. The induction of microtubule-dependent rounding and inhibition of proliferation after cytotactin binding are consistent with the conclusion that cytotactin is an endogenous cell surface modulatory protein. In this respect, it shows some effects similar to those of the lectin Con A (4, 28).

A number of studies have demonstrated a correlation between the extent of cell flattening and pH_i (29–32), suggesting that the regulation of pH_i is important for anchorage-dependent growth. Some authors have postulated that transformed cells escape anchorage dependence, allowing them to grow in suspension by their ability to maintain a relatively alkaline pH_i even when round (30). Other studies have established a link between intracellular alkalization and stimulation of cell growth by a variety of growth factors (reviewed in refs. 20 and 21). For example, in capillary endothelial cells, cell shape, pH_i , and DNA synthesis are all correlated (32); similarly, growth factor stimulation of thymidine incorporation can be inhibited by preventing intracellular alkalization by using amiloride to inhibit the Na^+/H^+ antiporter (reviewed in refs. 20 and 21).

In the present studies with cytotactin, although cells were initially maintained in a more rounded state, the correlation between rounding and inhibition of proliferation was not absolute. Consistent with this, in the presence of cytotactin, fibroblasts pretreated with nocodazole rapidly flattened but

DNA synthesis was still inhibited. Thus the modulating effects of cytotactin on cell rounding and DNA synthesis appear to occur in parallel rather than in causal sequence. This separation of rounding and proliferative effects is similar to recent results with the extracellular matrix protein SPARC, which causes endothelial cell rounding and inhibits cell proliferation (33). A fragment of SPARC that does not cause cell rounding nevertheless retained its ability to inhibit thymidine incorporation, whereas another fragment that prevented cell spreading had no effect on proliferation (33). In addition, the separation of cell shape from effects on pH_i is suggested by recent studies (34) that indicate that it is integrin clustering, and not cell shape changes *per se*, that is related to these phenomena.

Cytotactin was able to inhibit cell proliferation initiated by several different growth factors or by the tumor promoter PMA. One possible explanation for these effects is that cytotactin may bind to the growth factors and thereby inhibit their mitogenic activity. This possibility has not been formally ruled out and deserves investigation. Several lines of evidence, however, suggest that growth factor binding is not the mechanism of cytotactin effects on proliferation. In each case, cytotactin was able to inhibit the maximal (for 3T3 cells) stimulatory concentrations of the growth factors, and increasing the growth factor concentrations 10-fold did not reverse the cytotactin effects. Moreover, the growth factors used are of quite varied primary structure and have distinct receptors, and cytotactin also inhibited thymidine incorporation stimulated by PMA, which acts by altering protein kinase C activity and not directly on surface receptors. These results raise the question as to whether cytotactin has effects on specific second-messenger pathways. Preliminary results have indicated that the presence of cytotactin has little to no effect on a number of growth factor-stimulated second messengers. This suggests that a pathway independent of these changes, such as one involved in the changes in pH_i , may be the main one in mediating the growth-inhibitory effects of cytotactin. Many intracellular mechanisms have been shown to be affected by pH_i (reviewed in ref. 21), including gap junctional communication and endocytosis (35) as well as protein trafficking in polarized cells (36), any of which could contribute to subsequent effects on cell proliferation. The direct binding of cytotactin to, or interference with the function of, the Na^+/H^+ antiporter is also not excluded. Whatever the mechanism, cytotactin-mediated local pH_i changes may be a significant mechanism contributing not only to the inhibition of DNA synthesis described here but also potentially to other effects of cytotactin such as its ability to inhibit neurite outgrowth and cellular migration (9, 17, 37, 38).

The effects of cytotactin on cell rounding and DNA synthesis focus attention on the mechanism of cytotactin action at the cell surface during development. So far, little progress has been made in identifying a cytotactin receptor, although binding dependent on the Arg-Gly-Asp sequence has been described (10, 13, 39). Several possible mechanisms of cytotactin action deserve consideration, including competition by cytotactin for fibronectin binding to its receptor or the possibilities that cytotactin binding to fibronectin itself may inhibit integrin-mediated cell binding to fibronectin or that cytotactin binding to a unique surface receptor modulates fibronectin receptor binding or clustering (34). The latter possibility would be consistent with the suggestion that cytotactin is a natural modulator. For example, the binding of cytotactin to its cell surface receptors might interfere with a fibronectin-induced integrin clustering. Integrin clustering has been shown to increase pH_i , and thus receptor clustering may be, at least in part, the functional equivalent of cell flattening on fibronectin (34).

An earlier study indicated that cytotactin/tenascin stimulated cell growth of primary mammary tumor cells (40). These appeared to be highly transformed cells that were unable to grow on other substrates such as type IV collagen or fibronectin. It is possible that different cell types may respond differentially to substrate molecules and that this would affect their subsequent responses to growth factors, as has been suggested for the response of embryonal carcinoma cells to activin and bFGF (41). These results focus attention on the need for studies of the responses of different types of cells to cytotactin as well as on possible differences between soluble and substratum-bound cytotactin, which have been shown to have opposite effects on neurite outgrowth (38).

The observations described here should prompt intensified searches for cytotactin receptors at the cell surface. They also should provoke investigations into the linkage of cytotactin binding with cytoskeletal changes as well as comparisons of cytotactin effects with those (1, 4) of Con A. A final point is worth noting: cytotactin is an unusual morphoregulatory molecule of the extracellular matrix and its expression may be regulated by homeotic or other pattern-forming genes (42). Cytotactin is therefore likely to be important in regulating the primary processes of development—particularly division, migration, and cell shape change—that act in a place-dependent fashion during embryogenesis, regeneration, tumorigenesis, and wound healing.

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