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Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein

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ABSTRACT **To examine the role of gap-junctional intercellular communication in controlling cell proliferation, we have transfected C6 glioma cells with connexin 43 cDNA. The growth of transfected clones was dramatically reduced compared with nontransfected glioma cells. To further characterize the role of gap junctions in controlling proliferation, we have examined the growth of C6 cells cocultured with transfected cells overexpressing connexin 43. Although C6 cells grew at their normal rate when cocultured with nontransfected C6 cells, when cocultured with connexin 43-overexpressing cells they displayed a dramatic reduction in growth rate. Furthermore, a significant, dose-dependent reduction in cell proliferation was noted when C6 cells were cultured in medium conditioned by transfected cells. This effect correlated with the level of connexin 43 expression. These results suggest that the decreased cell proliferation rate of transfected cells and C6 cells cultured with them is due to the secretion of a growth inhibitory factor(s) and that the secretion of this factor may be linked to the level of gap junctional intercellular communication.**

Intercellular communication via gap junctions has been shown to play a role in the regulation of cell proliferation and subsequent differentiation (1). In fact, there is good evidence that an interruption of this communication pathway is one of the steps in malignant transformation (for review, see ref. 2): a variety of chemical agents that transform cells *in vitro* reduce intercellular communication. Conversely, the growth of transformed cells can be inhibited when they come into contact with normal cells, and this is correlated with the assembly of gap junction channels between the two cell types (3). Although several studies have shown that the growth of cancer cells can be controlled by surrounding normal cells (3–7), the mechanism(s) by which this occurs is complex, with evidence for a role of cell-to-cell contact (1–3, 8–12), as well as production of a soluble growth inhibitory factor(s) (7, 13). With reference to the first of these, evidence has been accumulating for the involvement of intercellular coupling via gap junctions (1, 2). We recently reported that C6 glioma cells exhibit marked reduction in expression of connexin 43, a gap junction protein, and are very weakly coupled (14), whereas transfection of these cells with connexin 43 cDNA restores intercellular coupling and reduces cell growth (15). By coculturing C6 glioma cells and connexin 43-expressing transfectants, we now report a marked reduction in growth of the C6 cells. Because medium conditioned by these transfected cells also reduced cell proliferation, there may be a relation between gap junctional coupling and the secretion of soluble growth-regulatory factors.

MATERIALS AND METHODS

Cell Culture and Proliferation. The C6 glioma cell line (16) (American Type Culture Collection) was established in monolayer culture in Dulbecco's minimum essential medium (DMEM) (GIBCO) supplemented with 10% fetal calf serum (FCS), penicillin G (100 units per ml), and streptomycin (100 μg/ml). Two connexin 43-transfected C6 clones, Cx43-13 and Cx43-14, were also cultured under similar conditions. Details concerning the transfection of C6 cells have been described (15, 17).

To examine cell proliferation, 1.5×10^6 cells were plated in DMEM/10% FCS in 60-mm Petri dishes. Culture medium was changed every 2 days. Cells in triplicate samples were counted with a hemocytometer at daily intervals.

Cocultures of C6 and Cx43-13 Cells. For cocultures, C6 cells were dissociated with 2 mM EDTA, suspended in DMEM, pelleted, and resuspended in 2 μM PKH26-GL (Zynaxis, Malvern, PA) (2×10^7 cells per ml) for 4 min (18). An equal volume of FCS was added after 1 min followed by 3 vol of DMEM/10% FCS, and several washes in DMEM/10% FCS. Labeled C6 cells were plated with a 40-fold excess of unlabeled C6 or Cx43-13 cells. The number of labeled cells in duplicate plates was counted in 8–10 random fields with a Zeiss Axiophot fluorescence microscope equipped with a 40× objective (0.28-mm^2 per field).

Dye Coupling. Functional intercellular coupling via gap junctions was assessed by intracellular injection of low-molecular-weight fluorescent dye. Labeled C6 cells were cocultured with unlabeled C6 or Cx43-13 cells as described above. After 2 days in culture, single PKH26-GL-labeled C6 cells, identified by their red fluorescence, were injected with 6-carboxyfluorescein (10 mM in distilled water, pH 7.0; Eastman Kodak), by using a continuous train of hyperpolarizing current pulses of 2–6 nA (200-ms duration, 1 per s). The quality and stability of electrode penetration were monitored by recording cell membrane potential during dye injection as described (14).

Growth in Conditioned Medium. To examine the effect of conditioned medium on cell growth, 3×10^5 C6 cells were plated in 60-mm dishes. The following day, the medium was replaced with a 1:1 mixture of DMEM/10% FCS and medium conditioned for 24 hr by C6, Cx43-13, or Cx43-14 cells, which were 80% confluent. Cells were counted as described above, and doubling times were determined when cell growth was in logarithmic phase.

To determine whether the effect of conditioned medium was reversible, C6 cells were initially cultured in clone Cx43-13-conditioned medium. After 2 days, this medium was replaced with unconditioned medium in half of the plates and with fresh Cx43-13-conditioned medium in the other half. Proliferation was monitored over several days.

The dose-dependent effect of conditioned medium was investigated with Cx43-13 cells cultured for 18 hr in DMEM without serum. This conditioned medium was collected and centrifuged at 3000 × *g* for 10 min. The supernatant was used undiluted (1×), or diluted 1:1 (0.5×) or 1:3 (0.25×) with fresh

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Abbreviations: FCS, fetal calf serum; DMEM, Dulbecco's minimum essential medium.

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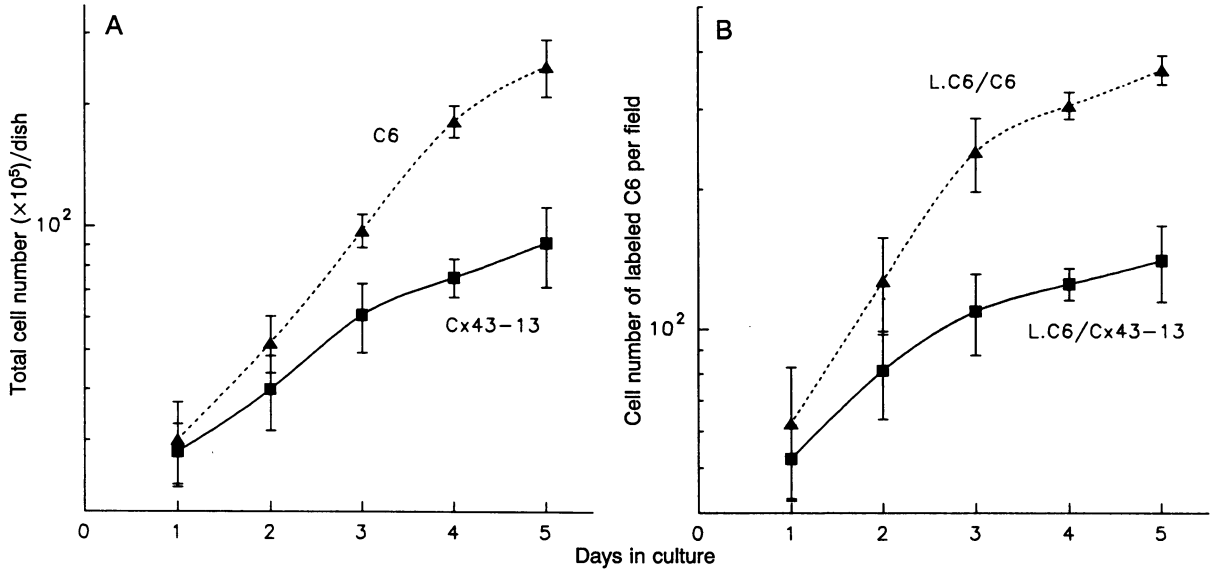

FIG. 1. (*A*) Growth rate of C6 glioma cells and transfected clone Cx43-13 cells. Significant difference in cell number was obvious by day 3 in culture. (*B*) Coculture of labeled C6 (L.C6) with unlabeled C6 (C6) did not alter growth rate, whereas coculture with Cx43-13 cells (L.C6/Cx43-13) significantly reduced the growth of C6 cells.

medium, and fresh FCS was added (to 10%). These various dilutions of conditioned medium were added to 3×10^5 C6 cells in triplicate 60-mm plates, and the cells were counted over several days as described above. Conditioned medium was replaced every 2 days.

RESULTS

Cultures of C6 glioma cells or a C6 clone that had been transfected with connexin 43 cDNA were set up for cell growth analysis. This clone, Cx43-13, has previously been shown to express a 50-fold increase in connexin 43 mRNA (15) and an 8-fold increase in connexin 43 protein (16). The Cx43-13 cells were highly coupled and displayed membrane potentials of approximately −50 mV to −60 mV. Determination of the growth rate revealed that clone Cx43-13 cells grew more slowly than nontransfected C6 cells (Fig. 1*A*).

To determine whether transfected cells displaying a high degree of intercellular coupling could influence the growth of tumor cells, nontransfected C6 cells were cocultured with

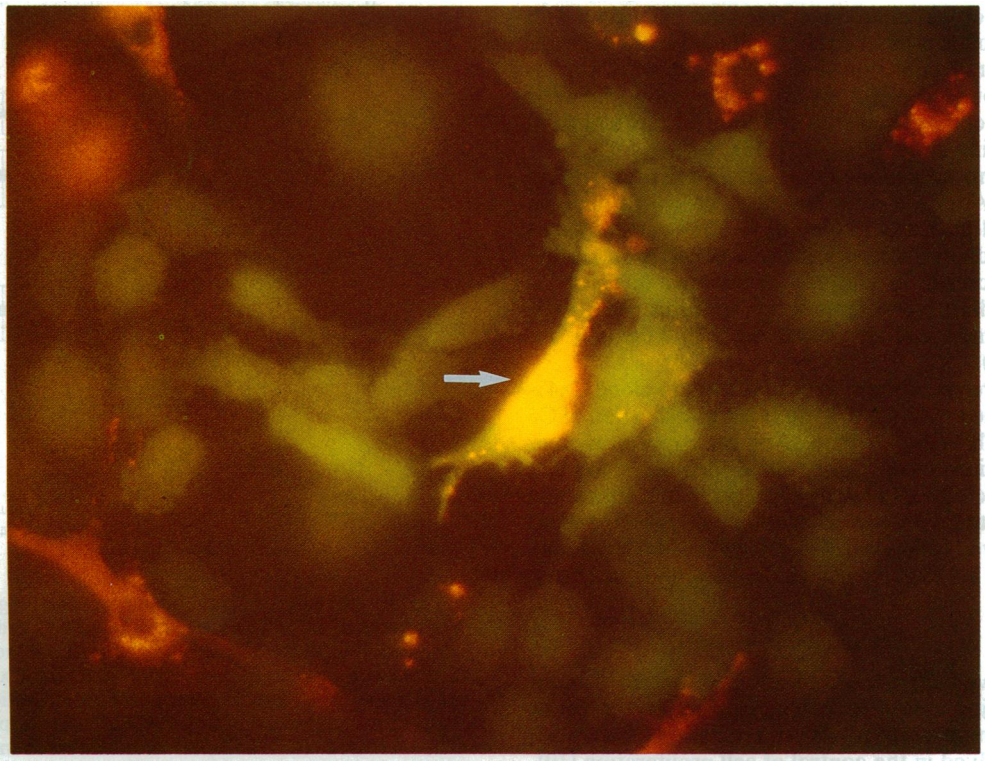

FIG. 2. PKH26-GL-labeled C6 cells were cocultured with unlabeled C6 or clone Cx43-13 cells. Although no passage of dye was seen from C6 cells cocultured with unlabeled C6 cells (data not shown), there was extensive dye passage from C6 cells cocultured with Cx43-13 cells. Arrow indicates the C6 cell initially injected with dye in this field. (×460.)

clone Cx43-13 cells. To distinguish C6 cells from the clone Cx43-13 cells, the C6 cells were labeled with the lipophilic dye PKH26-GL before coculturing. The dye itself did not affect cell proliferation. Under these conditions, C6 cells grew at the same rate as clone Cx43-13 cells, with a doubling time of ≈42 hr (Fig. 1*B*). In contrast, labeled C6 cells cocultured with unlabeled C6 cells had a doubling time of 19 hr.

To assess whether these C6 cells were coupled via gap junctions to clone Cx43-13 cells, single cells were injected with the low-molecular-weight dye 6-carboxyfluorescein. Labeled cells could be readily identified and targeted for dye injections. PKH26-GL-labeled C6 cells cocultured with unlabeled C6 cells rarely exhibited passage of dye from single injected cells. In contrast, PKH26-GL-labeled C6 cells became extensively coupled to unlabeled clone Cx43-13 cells when these cells were cocultured (Fig. 2). Dye was observed to spread from single injected C6 cells to 20–30 surrounding cells within 3 min. When unlabeled Cx43-13 cells were injected with dye, fluorescence rapidly spread to surrounding cells, including PKH26-GL-labeled C6 cells cocultured with them.

To test whether cell-to-cell contact was necessary for this reduced proliferation, C6 cells were grown in medium conditioned by C6 cells or clone Cx43-13 cells. Analysis of cell proliferation indicated that medium conditioned by clone Cx43-13 also had an effect on the growth of C6 cells (doubling time of 23 hr), whereas there was no effect with C6-conditioned medium (doubling time of 14 hr) (Fig. 3*A*). This effect of Cx43-13-conditioned medium was not mediated through an increase in intercellular communication via gap junctions because there was no effect on the level of dye-coupling between C6 cells (unpublished observation).

If the growth inhibitory effect of conditioned medium was due to the extent of gap-junctional coupling, it should correlate with the level of connexin 43 expression. Because we had previously obtained several stable clones that expressed the transfected connexin 43 cDNA to variable degrees (15), we were able to obtain conditioned medium from cells with different levels of gap-junctional coupling. Medium conditioned by a moderate connexin 43-expressing clone (Cx43-14) had a less pronounced effect on C6 proliferation (doubling time of 17 hr) in comparison with conditioned medium from the high-expressing clone (Cx43-13) (Fig. 3*A*).

The effect of this conditioned medium on cell proliferation was reversible. After C6 cells had been grown in medium conditioned by clone Cx43-13 for 2 days, they were cultured in normal C6 medium. Although the rate of cell proliferation continued to decrease in cultures maintained in Cx43-13-conditioned medium, proliferation in cultures that were switched to normal unconditioned medium gradually increased (Fig. 3*B*).

When serum-free medium was used to culture clone Cx43-13, this conditioned medium still inhibited proliferation of C6 cells (Fig. 3*C*). This effect depended on the concentration of conditioned medium used. Conditioned medium alone had the most dramatic effect (doubling time of 36 hr). When this medium was diluted with normal medium, a dose-dependent effect was observed [dilution 1:1 (0.5×) gave a doubling time of 18 hr; dilution 1:3 (0.25×) gave a doubling time of 14 hr].

DISCUSSION

Many studies have supported the view that increased mitogenesis is a major factor in carcinogenesis (4). Because intercellular communication via gap junctions has been shown to be involved in the control of cell proliferation (19), it is not surprising that evidence has linked the loss of intercellular coupling with tumorigenesis (2). To directly examine this correlation, we have initiated a series of studies

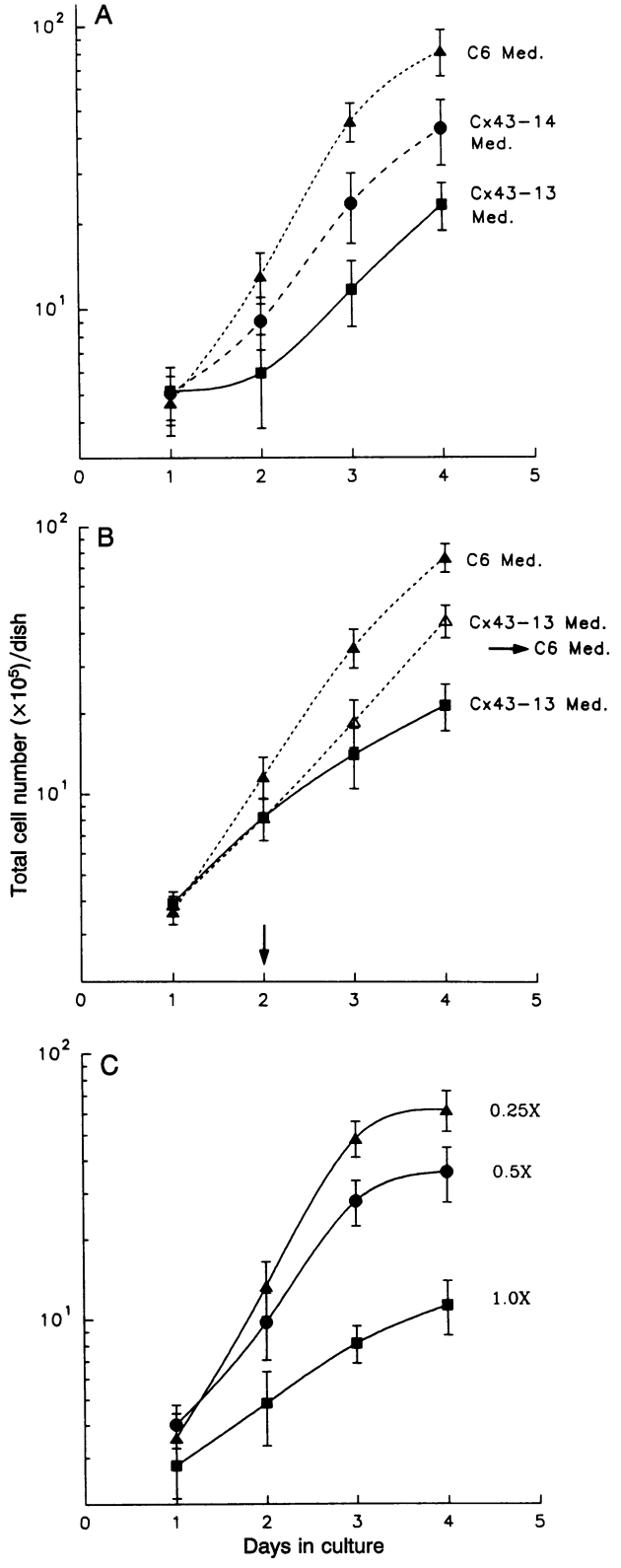

FIG. 3. (*A*) Effect of conditioned medium on growth of C6 cells. The growth of C6 cells was most dramatically reduced by Cx43-13-conditioned medium, whereas Cx43-14-conditioned medium had an intermediate effect. (*B*) Reversible effect of Cx43-13-conditioned medium on C6 cell growth. When Cx43-13-conditioned medium was replaced with C6-conditioned medium (arrow), proliferation of C6 cells increased. (*C*) The effect of conditioned medium on C6 cell growth was dose dependent. Undiluted conditioned medium (1×) dramatically reduced the growth of C6 cells. In contrast, when this conditioned medium was diluted with normal medium (0.5×, 0.25×), a dose-dependent decrease in doubling time was seen.

transfecting connexin 43 cDNA into C6 glioma cells that express relatively low levels of this gap junction mRNA and protein, are poorly dye-coupled, and exhibit rapid growth (14). These transfected cells exhibit increased levels of connexin 43 mRNA and protein (15, 17), increased dye-coupling (15), and intracellular Ca^{2+} signaling that is directly related to the level of connexin 43 expression (20). The resulting proliferation of stable clones was inversely proportional to the degree of expression of the transfected cDNA (15). In addition, we have observed a similar reduction in the growth rate of transfected cells *in vivo*, both in the rat brain (21) and in nude mice (22).

Our present data clearly demonstrate that overexpression of connexin 43 in C6 glioma cells significantly reduces cell proliferation. There was no evidence of increased cell death in transfected cells (unpublished observation). This growth inhibition was also evident when C6 cells, which normally exhibit very little gap junctional intercellular coupling, were cocultured with connexin 43-expressing transfected clones. The C6 cells became highly coupled to transfected cells that express a high level of connexin 43. This coupling was coincidental with reduced proliferation, suggesting that transfected cells modulate the growth rate of C6 cells, possibly via hypothesized transmission of cytoplasmic growth-controlling signals through gap junctions (23). On the other hand, medium conditioned by the connexin 43-overexpressing cells also repressed C6 cell growth, whereas C6-conditioned medium did not alter proliferation. This result suggests that cell-to-cell contact is not necessary for the observed growth inhibition. Gap junctional coupling may exert an effect on cell proliferation by influencing the secretion of a soluble growth inhibitor. Several reports have described a growth-inhibitory effect of fibroblast-conditioned medium on a variety of normal and tumor cell types (7, 13, 24–26). In preliminary studies, we have found that conditioned medium from connexin 43-transfected C6 cells also inhibits the growth of several other tumor cell lines (22).

Glioma cells produce a variety of growth factors, many of which have been implicated in autocrine mitogenic activities (27, 28). Several of these growth factors have been shown to decrease gap junctional intercellular communication (29). However, the role of gap junctions in controlling cell growth through altering growth factor pathways has not been investigated. It is possible that gap junctional communication may play a role in regulating the production of one or more factors involved in cellular growth control.

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