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Conditioned medium from activated spleen cells supports the survival of rat retinal cells *in vitro*

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Abstract

Cytokines are a heterogeneous group of molecules that have been associated with several functions in the nervous system, such as survival and differentiation of neuronal and glial cells. In the present study, we demonstrated that conditioned medium from spleen cells activated with concanavalin A increased neuritogenesis and survival of retinal cells, as measured by biochemical and morphological criteria. Our data showed that conditioned medium induced a five-fold increase in the amount of protein after 120 h *in vitro*. This effect was not inhibited by the blockade of voltage-dependent L-type calcium channels with 5.0 µM nifedipine. However, the use of an intracellular calcium chelator (15.0 µM BAPTA-AM) inhibited this effect. Our results support the idea that factors secreted by activated lymphocytes, such as cytokines, can modulate the maintenance and the differentiation of rat retinal cells *in vitro*, indicating a possible role of these molecules in the development of retinal cells, as well as in its protection against pathological conditions.

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The growth, differentiation, survival, and death of neuronal cells can be modulated by specific molecules produced during the normal development of the nervous system (1). Over the last few years it has been demonstrated that molecules produced by cells of the immune system can affect different neuronal populations as well as glial cells (2,3). In addition, glial cells have been shown to produce and release at least some of these molecules (4).

Cytokines are molecules with pleiotropic effects originally described as having immunoregulatory functions (5,6). However, it is their action on the nervous system as neurotrophic and differentiation factors that is presently well known (2,6). Various effects of cytokines on the nervous system have been described including neuronal survival and differentiation, neurite outgrowth, synaptic plasticity, modulation of neurotransmitter systems and hormonal regulation (1,3,7). Cytokines are a heterogeneous group of molecules including trophic factors, interleukins and other factors with the ability of inducing differentiation of specific cell populations (5,7). Cytokines also have an important role in inflammatory processes and in

conditions following neuronal injuries in adult life (2,5,6).

To test the action of cytokines on neurons in the developing central nervous system, we have studied a particularly well-characterized region, the neural retina. In the present study, we investigated the effect of conditioned medium produced by spleen cells activated with concanavalin A (ConA) on retinal cells in culture. When spleen cells are activated with ConA they produce a broad spectrum of growth-promoting factors such as colony-stimulating factors, interleukins, and a nerve growth factor-related molecule (8-10). The present results suggest that these cytokines can promote neuronal survival and neurite outgrowth in dissociated cultures of newborn rat retinal cells, as evaluated by both morphological criteria and protein assays. Our data indicate that these molecules may have important roles in neuronal and glial development.

Primary cultures were prepared using procedures previously described (11). Briefly, Lister Hooded rats at postnatal day 1 or 2 were killed by decapitation and the retinas were dissected free from scleral tissue and pigmented epithelium in calcium- and magnesium-free balanced salt solution (CMF). The retinas were incubated in CMF containing 0.1% trypsin (Worthington, Freehold, NJ) for approximately 17 min at 37ºC. The cells were mechanically dissociated using a polished Pasteur pipette and added to plastic Petri dishes (35 mm) previously coated with poly-L-ornithine (50 µg/ml; Sigma Chemical Co., St. Louis, MO) at a plating density of 1.0×10^6 cells/dish in complete culture medium (199; Gibco, Gaithersburg, MD) containing 2 mM glutamine, 100 µg/ml streptomycin + 100 U/ml penicillin (Sigma) and 5% fetal calf serum. The cultures were then maintained at 37ºC in an atmosphere of 5% CO_2/95% air for several days. The conditioned medium (CM) obtained from ConA-activated spleen cells was prepared by the method of Gozes et al. (9) modified in our laboratory. Briefly, adult Lister Hooded rats were killed by ether asphyxia and the spleens were dissected in CMF solution. The single-cell suspension was obtained by passing spleens through a 50-gauge plastic mesh. The culture medium containing the cells was left to rest for 1 h on plastic dishes (35 mm) in order to allow the attachment of macrophages. The supernatant containing the non-adherent cells was aspirated, centrifuged (2000 *g* for 5 min), and added at a plating density of 5.0×10^6 cells/ml to complete culture medium supplemented with 2-mercaptoethanol (25 µM) and ConA (5.0 µg/ml) for approximately 12 h at 37ºC in an atmosphere of 5% CO_2/95% air. The supernatant thus obtained was again centrifuged at 2000 *g* for 10 min and the CM was sterilized by filtration through a 0.2-µm membrane and kept at 4ºC. After the maintenance of retinal cells in culture for specific times, the morphology of the culture was analyzed and the total amount of protein in the cultures quantified by the method of Lowry et al. (12).

Figure 1 shows that CM kept cultured retinal cells viable for at least 120 h whereas pre-coating of culture dishes with CM did not elicit the same effect, suggesting that only soluble factors are involved (data not shown). Similarly, treatment with ConA alone did not affect the viability of retinal cells (data not shown), while CM maintained the survival of retinal cells. The action of CM on the morphology of retinal cells was first observed *in vitro* after 48 h (Figure 1B). Some changes in cell morphology were already observed after this time, with an increase in the number of small clusters of neuronal and glial cells (Figure 1B). The number of cell-bearing processes was also increased when compared with control cultures (Figure 1A,B). However, after 48 h the amount of protein in cultures treated with CM (103.51 ± 4.11 µg protein/Petri dish) was not different from that observed in control cultures (109.45 ± 4.82 µg protein/Petri dish). After 120 h of incubation with CM the

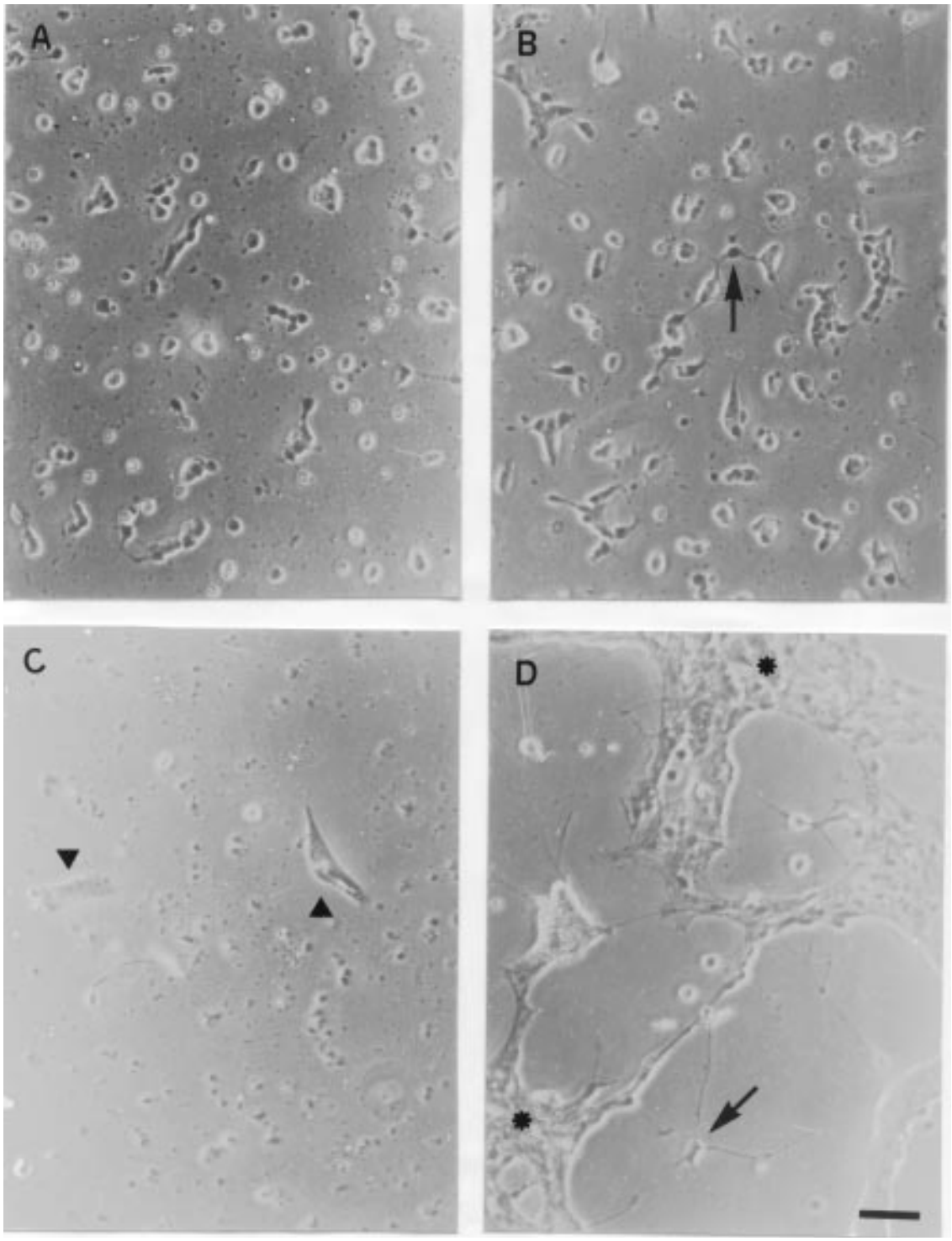

Figure 1 - Phase contrast photomicrographs of retinal cells *in vitro*. Cells were dissociated from neonatal rat retina and cultured for 48 (A and B) and 120 h (C and D). *A* and *C*, Control cultures; *B* and *D*, cultures maintained in the presence of conditioned medium (1:10). The plating density was 1.3 x 10^6 cells/dish. Note the presence of isolated neuronal cells with long neurites (arrows) and flat-shaped glial cells (arrowheads) in the cultures. Extensive clusters of neuronal and glial cells are indicated by asterisks. Magnification bar for all photomicrographs: 50 µm.

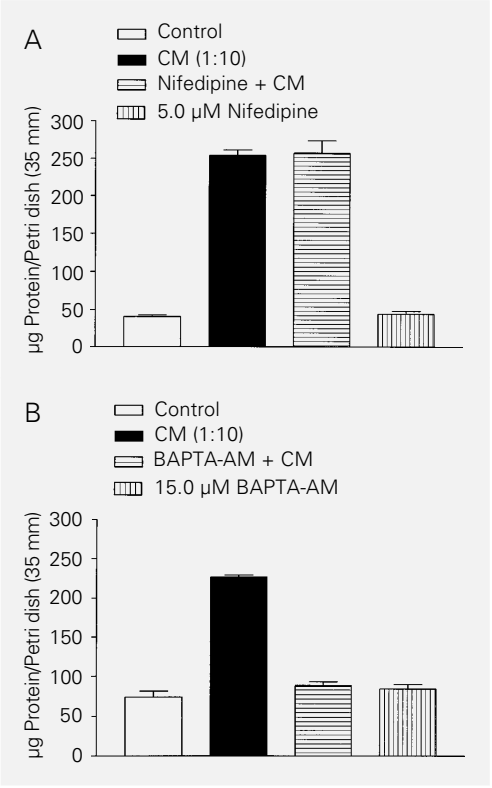

Figure 2 - Histograms showing the protein content of retinal cultures. *A*, The cells were incubated for 120 h with control medium (control), conditioned medium (CM; 1:10), 5.0 µM nifedipine or nifedipine + CM. *B*, The cells were incubated for 96 h with control medium (control), conditioned medium (CM; 1:10), 15.0 µM BAPTA-AM or BAPTA-AM + CM. Data are reported as the mean ± SEM of 3 independent experiments, each performed in triplicate.

presence of large clusters of neuronal cells supported by glial cells was clearly visible. Neuritogenesis, neuronal survival as well as glial cell proliferation were highly stimulated by CM (Figure 1D). The cells maintained in the absence of CM died within 120 h as observed by morphological criteria, i.e., shrinkage of the body of neuronal cells and disruption of neuronal processes (Figure 1C). In the control cultures there were only glial cells that took on a flat shape characteristic of this cell type (Figure 1C).

As shown in Figure 2A, CM also increased the amount of protein compared with control cultures. The increase in protein in treated cultures reflects not only the proliferation of glial cells but also the survival of neuronal cells. In fact, the use of the antimitotic agent 2-fluorodeoxyuridine (20.0 µM) did not block the survival or neuritogenesis of neuronal cells stimulated with CM (data not shown).

The maintenance of retinal cells in culture by the treatment with CM was dose-dependent and heat-sensitive, and was observed at all plating densities studied (data not shown). Moreover, the blockade of voltage-dependent L-type calcium channels with nifedipine (5.0 µM) did not inhibit the action of CM on retinal cells (Figure 2A). On the other hand, it appears that the cytoplasmic calcium levels were essential for the effect of CM since BAPTA-AM (15.0 µM), an intracellular calcium chelator, was efficient in blocking the increase in the amount of protein (Figure 2B).

The regulation of cytoplasmic calcium levels has been described in different events during the development, maintenance, and degeneration of neuronal cells. This intracellular messenger actively participates in several signaling pathways in the membrane and in the cytoplasm. Indeed, the survival of developing neuronal cells deprived of trophic factors in culture has been related to the control of cytoplasmic levels of free calcium (13). Our results show that this second messenger is important for the action of CM since the addition of BAPTA-AM completely abolished this action. Nevertheless, the blockade of voltage-dependent L-type calcium channels did not alter the effect of CM, even though the influx of calcium through these channels is known to play a key role in the survival of other neuronal cell types (13,14).

While most of the reported effects of cytokines are on non-neuronal cells, there is also evidence that they may directly act on neurons (3,4,15,16). In addition, the early time course of the expression of some cytokines suggests that these molecules may have important effects on neuronal differentiation (1,7,16,17).

Although the retina has been extensively studied by anatomical, histological and biochemical methods, there is still very little information concerning the mechanisms that control its development leading to cellular differentiation. Strong evidence suggests that the development of retinal cells is regulated by cell-cell contacts and by diffusible mol-

ecules (4,11,18).

In general, cytokines produced and secreted by lymphocytes are related to inflammatory and degenerative processes (2,5,6). It is well known that lymphoid cells also have the ability to secrete growth-promoting factors that participate in a pleiotropic manner in the development of the nervous system and in its protection against pathological conditions (1,9,15,17). Our results support the idea that molecules secreted by activated lymphocytes can modulate the maintenance and differentiation of rat retinal cells *in vitro*, indicating a possible role of these molecules in the development of retinal cells.

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