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INSULIN-LIKE GROWTH FACTOR 1 SUPPORTS EMBRYONIC NERVE CELL SURVIVAL

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Summary: Insulin-like growth factor 1 (IGF-1) is shown to support the long term survival of embryonic chicken central nervous system neurons cultured in a defined medium. This trophic activity for IGF-1 was discovered as the result of its presence as a contaminant in bovine serum albumin, a reagent frequently used in primary cultures of neuronal tissue. The observation that IGF-1 has cell survival activity may explain the high level of IGF-1 receptors found in embryonic brain.

Although the receptors for insulin and insulin-like growth factor 1 (IGF-1) are abundant in the central nervous system (CNS) (1-3), the role of IGF-1 in the brain is not clear. The observation that the concentration of IGF-1 receptors is higher in the developing CNS than in adult tissue (4-6), suggests that IGF-1 may play a role in the development of the nervous system. In addition, IGF-1 promotes neurite outgrowth in neurons cultured from the brain, spinal cord, sensory, and sympathetic ganglia, as well as neuroblastoma cells (7-9). These data suggest a role for IGF-1 in nerve cell growth and differentiation.

Bovine serum albumin (BSA) is a frequent component of defined culture media used for primary cultures of neuronal cells (see for example 10, 11). When BSA is added to culture medium, it is presumed that it acts as a protein supplement as opposed to supplying specific

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Abbreviations: BSA, bovine serum albumin; CNS, central nervous system; EMB, Eagle's basal medium; IFG-1, insulinlike growth factor 1.

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hormones to cells. We observed, however, that when BSA is added to dissociated cultures of embryonic chick cortical neurons, there was a pronounced dose-dependent survival of the cells. It was asked if this apparent neurotrophic effect of BSA was due to the albumin per se or due to a contaminant with CNS cell survival promoting activity. The following paragraphs show that the neurotrophic activity in commercial preparations of BSA is due to an insulin-like growth factor. Because there is a high density of IGF-1 receptors in the developing nervous system, IGFs may have an important neurotrophic role in CNS development.

Materials and Methods

Eight day embryonic chick forebrain was dissociated in Eagle's Basal Medium (EBM) supplemented with 26.4 mM $NaHCO_3$, 33.3 mM D-glucose, 2 mM L-glutamine, 100 units per ml of penicillin and 0.08% trypsin for 20 min at 37°C. Vogt and Dulbecco's modified medium (12) was not used because it contains pyruvate and pyruvate can prolong nerve cell survival in culture (13). The cells were then washed once with 1% ovalbumin to inactivate the trypsin and plated in EBM containing 5 µg/ml insulin, 5 µg/ml transferrin, 10^{-4}M putrescine, $2x10^{-8}$M progesterone, and $3x10^{-8}$M selenium as Na_2SeO_3 (14) into 6 mm Costar wells pretreated with polyornithine (30,000 MW) at 0.1 mg/ml in 15 mM borate buffer, pH 8.4. The plating density was 5,000 cells per well. Cells were fixed with 2% glutaraldehyde in EBM and the number of remaining cells assessed by visual inspection with phase contrast optics. In some cases viable cell number was determined by the method of Manthorpe et al. (15).

Gel electrophoresis in 20% acrylamide and 0.1% $NaDoSO_4$ was done according to Anderson et al., (16) and the proteins transferred to nitrocellulose by electroblotting and the ability of the transferred proteins to support cell survival determined by published procedures (17).

All reagents were purchased from Sigma, with the exception of recombinant human IGF-1 which was obtained from Amgen and the neutralizing monoclonal antibody against IGF-1 which was a generous gift of Dr. J.J. Van Wyk (18).

Results

When dissociated 8 day chick embryonic cortical neurons are cultured in the presence of 1% BSA in a chemically defined culture medium containing transferrin, insulin, putrescine, progesterone and selenous acid (14), a large fraction of the cells survive for extended periods of time, frequently over 30 days. However, in the absence of BSA, the cells die within 24 hrs. When the BSA in the culture medium is

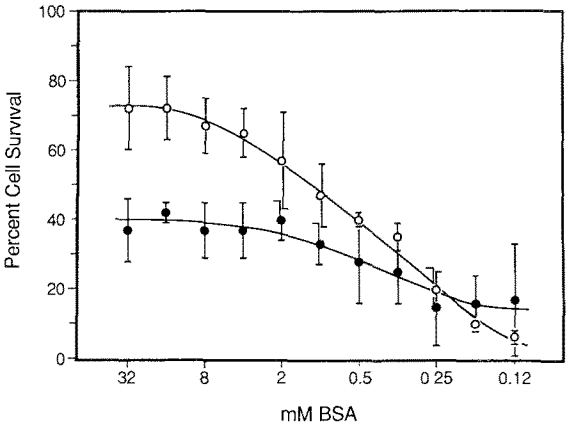

Figure 1. Dose response curve for BSA. Eight day embryonic chick cortical cells were plated at 5,000 cells per well as described in Materials and Methods. The number of live cells was determined on the indicated days. The data are plotted as the mean plus or minus the standard error of 5 cultures for each point. o--o, cell number after 1 day; •--•, cell number after 5 days.

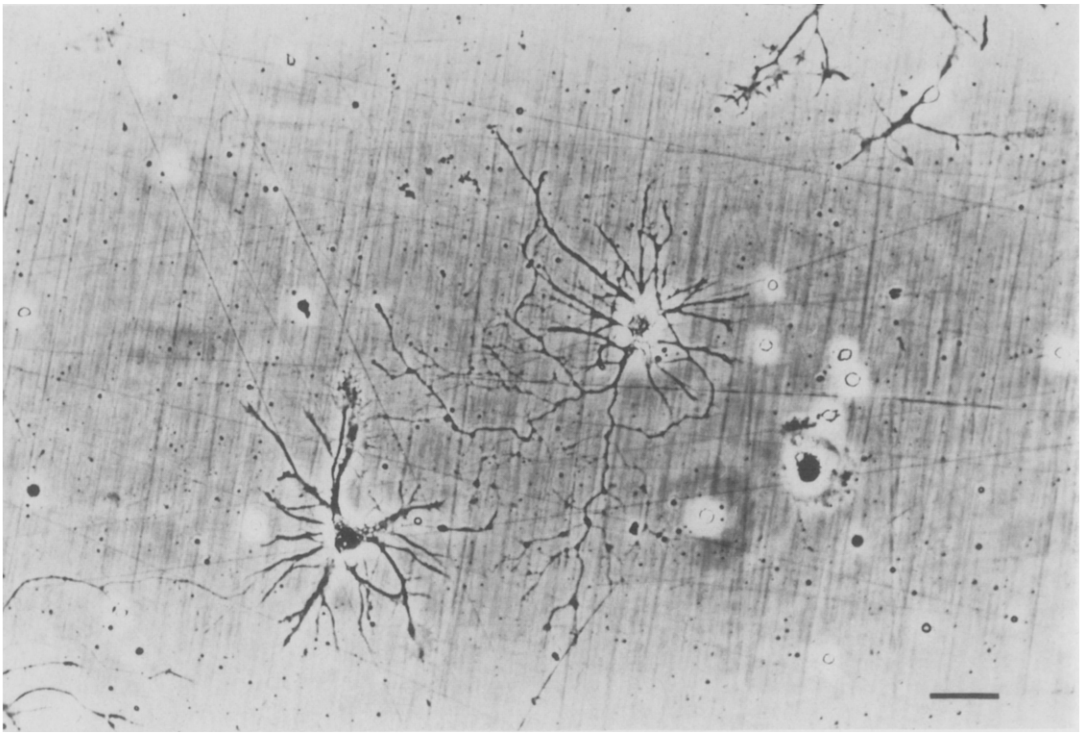

Figure 2. Cells after 7 days in culture in the presence of 0.5% BSA. Bar indicates 20 microns.

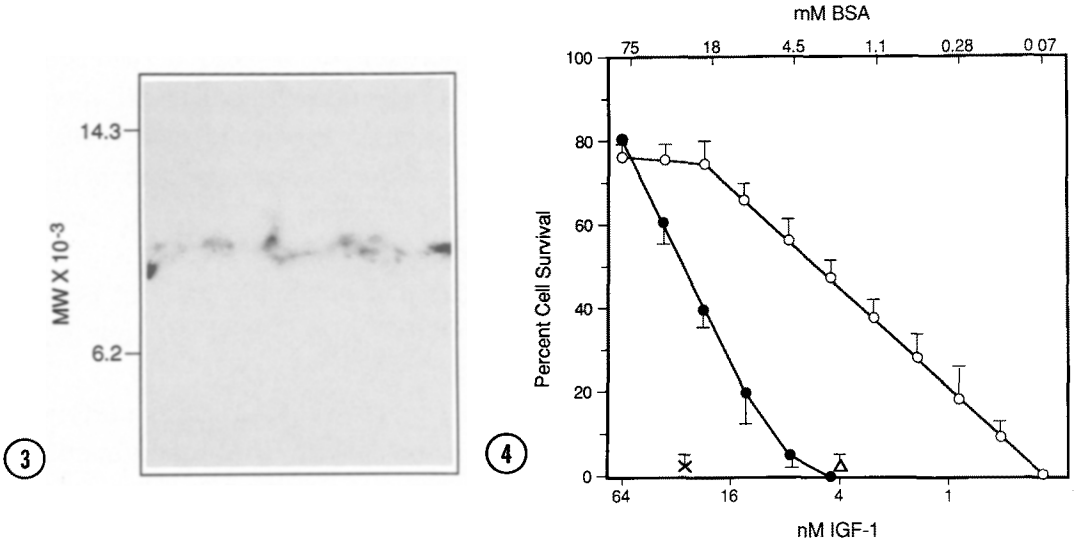

Figure 3. Cell-blot technique identification of BSA activity. One mg of BSA was electrophoresed on a 20% acrylamide gel containing 0.1% SDS and the proteins transferred to nitrocellulose. Cells were grown on top of the nitrocellulose without addition of BSA for 48 hours, and the living cells visualized with a vital dye (17).

Figure 4. Titration curve showing the trophic support of BSA and recombinant IGF-1 after 24 hours in vitro. Abscissa represents serial dilution of the indicated protein. In the case of BSA the starting concentration was 75 mM. For IGF-1, the corresponding value was 67 nM. The minimum protein concentration of neutralizing antibody (gift from Dr. J. J. Van Wyk) which completely blocked the half maximal effect of BSA was 11 ng/ml (Δ). The half-maximal effect of pure IGF-1 was neutralized by 15 ng/ml of the monoclonal antibody (X). The IGF-1 and BSA solutions were preincubated with the antibodies one hour at 37°C before addition to the cells. An unrelated monoclonal had no effect at 50 ng/ml. •---•, IGF-1; o--o, BSA.

serially diluted and the survival of cells determined after 1 and 5 days, there is a dose dependent response to BSA (Fig. 1). The only surviving cells are nerve as defined by their nerve-like morphology with extensive neurite branching (Fig. 2), and the fact that all cells stain positive for neurofilament protein, but not for glial fibrillary acid protein (data not presented). The above results suggest that either BSA or a contaminant in the commercial BSA preparations promotes the survival of the embryonic chick neurons. Three sets of experiments were done to distinguish between these alternatives.

Neurotrophic molecules such as ciliary neurotrophic factor support the viability of cells when transferred to nitrocellulose (17). To determine the approximate molecular weight of the cell survival activity of the BSA preparation, BSA was electrophoresed on a 20% acrylamide gel containing SDS, the proteins transferred to nitrocellulose, and then the nitrocellulose overlaid and incubated with embryonic brain cells. Two days later the nitrocellulose was gently washed and exposed to MTT, a metabolizable dye which turns black in the presence of living cells (17). Figure 3 shows that cell survival was enhanced by a protein of approximately 11,000 daltons; no survival was evident in the area of BSA mobility (70,000 daltons) or elsewhere in the gel. Therefore BSA per se is not responsible for neurotrophic support in these cultures.

Insulin-likelike growth factors are proteins of approximately 11,000 daltons, and are contaminates of commercial BSA preparations (19). Since embryonic CNS tissue contains high levels of IGF-1 receptors (4), the possibility that IGF-1 is the molecule in commercial BSA which supports nerve cell survival was examined. Figure 4 shows that like BSA, recombinant IGF-1 supports nerve cell survival in a dose dependent manner. A purified monoclonal antibody against IGF-1 (18) completely blocks the half maximal response to IGF-1 at 15 ng per ml (Fig. 4). If IGF-1 is the molecule in BSA which is responsible for the cell survival activity, then antibodies against IGF-1 should block the survival activity of BSA. Figure 4 also shows that anti-IGF-1 completely inhibits the half-maximal survival activity of BSA at 11 ng per ml. These data show that IGF-1 or a molecule antigenically related to it in BSA are able to support the survival of embryonic chick CNS neurons. Since the monoclonal against IGF-1 reacts poorly with IGF-2 (18), it is probable that IGF-1 is present in BSA.

Discussion

The above data show that IGF-1 supports the survival of embryonic cortical chick neurons. IGF-1 and its related family of molecules are found throughout the central nervous system along with their receptors (see for example, 20). The fact that IGF-1 receptor concentrations are higher in embryonic tissue suggests a role for IGF-1 in development (4, 5). Among the potential influences of IGFs in the developing nervous system are the stimulation of cell proliferation (21, 22) oligodendrocyte differentiation (23), and neurite formation (24, 25). The data presented above extend this list with demonstration that IGF-1 is able to promote the survival of embryonic CNS neurons.

The medium in which the embryonic CNS cells were grown contains $1x10^{-6}$M insulin, while the half-maximal survival response to IGF-1 in

the presence of insulin is $2x10^{-8}M$ (Fig. 4). These data are compatible with those showing that insulin interacts with the IGF-1 receptor about 100-fold less well than IGF-1 (see for example, 21). Since the high concentration of insulin alone in the culture medium is not sufficient to sustain the survival of 8 day embryonic chick CNS neurons, while the additional $2x10^{-8}M$ IGF-1 is sufficient, the IGF-1 receptors involved in mediating cell survival must have a much higher affinity for IGF-1 than for insulin.

These results are similar to those of Aizenman and de Vellis (24) who showed that IGF-1 could replace insulin in promoting the survival of rat cortical neurons. However, in the rat cultures 5 μg/ml of insulin alone was sufficient to keep the cells alive, while IGF-1 was required in addition to 5 μg per ml of insulin to cause the survival of embryonic chick neurons. This difference could be due to differences in the relative affinities for insulin and IGF-1 receptors for the two ligands in the different culture systems. Another possibility is that a seven-fold higher cell density was used in the rat cultures (22) than we used with the chick neurons. The high cell density could allow for a greater degree of trophic support between the cells themselves and lower the insulin requirement.

Fig. 4 shows that a monoclonal antibody against IGF-1 blocks the half-maximal effects of both recombinant IGF-1 and the component in BSA which is responsible for promoting nerve cell survival. The amount of antibody required to block this response was approximately the same (11 ng/ml for BSA and 15 ng/ml for pure IGF-1), suggesting that the BSA component which promotes survival is IGF-1. Even though the monoclonal antibody used in these experiments reacts approximately 20-fold less well with IGF-2 than with IGF-1 (18), our experiments do not formally establish that IGF-1 is the biologically active molecule in BSA. Although the only IGF component in commercial BSA preparations which has been unambiguously identified is IGF-2 (19), it is probable that BSA preparations contain both IGF-1 and IGF-2. However, since IGF-1 and IGF-2 have only a two-fold difference in their affinities for the IGF-1 receptor (26), the biological activities of both proteins are likely to be similar in promoting CNS nerve cell survival.

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