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Deficiency of a neuronal growth-sustaining factor in fibroblasts of patients with Alzheimer's disease

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Abstract

A previous report has shown a deficiency of a cholinergic differentiating factor in spent culture media in which Alzheimer's disease (AD) patient fibroblasts were grown (Kessler, 1987). We used a similar approach to investigate whether AD fibroblast-conditioned medium demonstrated central nervous system (CNS) neuronal growth sustaining properties. For these investigations we used cultured fetal murine telencephalic vesicle neurons. Control fibroblast-conditioned medium produced statistically significant neuronal survival as compared to AD fibroblast-conditioned medium or to nonconditioned medium. There was no statistically significant difference between AD fibroblast-conditioned medium and nonconditioned medium results. These results suggest that there may be a deficiency in AD of a CNS neuronal growth-sustaining factor.

Keywords: Alzheimer's disease; Fibroblasts; Growth factor; Tissue culture

1. Introduction

Kessler (1987) investigated the effects of Alzheimer's disease (AD) fibroblast-conditioned medium on the induction of choline acetyltransferase (CAT) in cultured neonatal rat superior cervical ganglion sympathetic neurons. He found that AD-conditioned medium increased CAT activity by only 38% of the control values. He did not observe any difference in neuronal numbers between the AD and control-conditioned mediums. These results indicated a deficiency of a cholinergic differentiating factor in AD.

The primary symptoms of AD are memory loss and cognitive decline due to central nervous system (CNS) neuronal death. Kessler's observations of a deficiency of a cholinergic differentiating factor in AD were based on effects on peripheral, sympathetic neurons, not on CNS neurons. It is important to determine whether in AD there is a loss of a CNS neuronal growth-sustaining factor. For this investigation we used techniques similar to Kessler's, but investigated survival of fetal murine CNS neurons.

2. Materials and methods

After obtaining signed, informed consent, 3-mm punch skin biopsies were aseptically obtained from two AD patients (a 74-year-old female and a 70-year-old male), who satisfied the NINCDS-ADRDA criteria for probable AD (McKhann et al., 1984) and their spouses (a 66-year-old female and a 76-year-old male). In the 74-year-old female AD patient, clinical symptoms had started 3 years previously, and in the 70-year-old male AD patient, 2 years previously. In both cases there was no family history of AD. At the time of biopsy all individuals were healthy and were taking no medications. The biopsy samples were minced with a scalpel into 1-mm fragments and were grown in tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The cultures were incubated at 37°C in a 94% air/6% CO_2 atmosphere. The medium was replaced every 4 days and the spent medium was stored aseptically at 20°C. The number of cells in the AD and control fibroblast cultures was the same. There were no passages of AD or of control fibroblasts.

Pregnant mice were sacrificed by cervical dislocation. Using sterile techniques, E16 normal fetuses were removed from the uterine sacks. Their telencephalic vesicles were

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dissected and homogenized through a coarse stainless steel sieve into a trypsin/EDTA solution for 2 min. The homogenates were washed several times in DMEM supplemented with 10% FCS and plated onto 53 separate collagen-coated coverslips at a plating density of 10^6 cells per ml. The batch of FCS used for the neuronal cultures was the same as that used for the fibroblast cultures. The coverslips were circular and fit precisely within the borders of sterile multiwell culture dishes. The cultures were incubated at 37°C in a 94% air/6% CO_2 atmosphere. The medium was replaced every four days. After two weeks, the medium in 19 cultures was replaced with a mixture of equal amounts of DMEM with 10% FCS and spent medium from the AD fibroblast cultures (9 cultures for the first AD patient and 10 cultures for the second AD patient). The spent medium was not filtered. Similarly, the medium in 18 cultures was replaced with a mixture of equal amounts of DMEM with 10% FCS and spent medium from the control fibroblast cultures (9 cultures for each of the two controls). In 16 cultures the original medium continued to be used. The medium combinations were replaced every three days.

Five small dots were placed on the bottom of each culture well. These dots were used as center marks to take phase-contrast photomicrographs of identical fields over time. Photomicrographs were taken before and after 1 and 2 weeks of treatment. Identical fields in each culture were photographed and used for analysis. The photographs were compared to determine whether a qualitative decrease or increase in neuronal numbers occurred. Quantification of cell numbers was not possible because cellular profiles extensively overlapped each other in the cultures.

Six additional murine CNS cultures were established and grown using the above described techniques. After two weeks the cultures were fixed in −20°C acetone for 5 min. After several rinses, two of the coverslips were incubated for 2 hr. in monoclonal antibody N210 (mabN210). The production and characterization of mabN210 has been described previously (Leclerc et al., 1985). MabN210 recognized specifically the highly phosphorylated 210 kDa subunit of neurofilaments and has been used for a number of immunohistochemical studies (Leclerc et al., 1985; Plioplys et al., 1986; Plioplys, 1987; Plioplys, 1988). After several rinses HRP-conjugated rabbit anti-mouse immunoglobulin (Dako, Inc.) was applied for 2 hr. The reaction product was visualized with 4-chloro-1-naphthol as colorant (Hawkes et al., 1982). Photomicrographs revealed patterns of neurofilament staining similar to previous reports (Asou et al., 1985). Preparations in which the primary antibody was omitted gave no staining. Similar techniques were followed to visualize the immunohistochemical expression of the 68 kDa neurofilament subunit and neuron specific enolase using monoclonal antibodies (Boehringer Mannheim). Two coverslips were stained with the anti-68 kDa neurofilament monoclonal antibody and two with the anti-neuron specific enolase monoclonal antibody.

For statistical analysis chi-square testing was used. The research protocols had been approved by hospital animal and human ethics review committees.

3. Results

After 2 weeks in culture, all of the coverslips had exuberant neuronal growth (Fig. 1A). The additional neuronal cultures that were stained with the 68 kDa and 210 kDa neurofilament subunit monoclonals and with the neuron specific enolase monoclonal all revealed extensive neuronal immunohistochemical reactivity: essentially all neuronal profiles were stained (not illustrated).

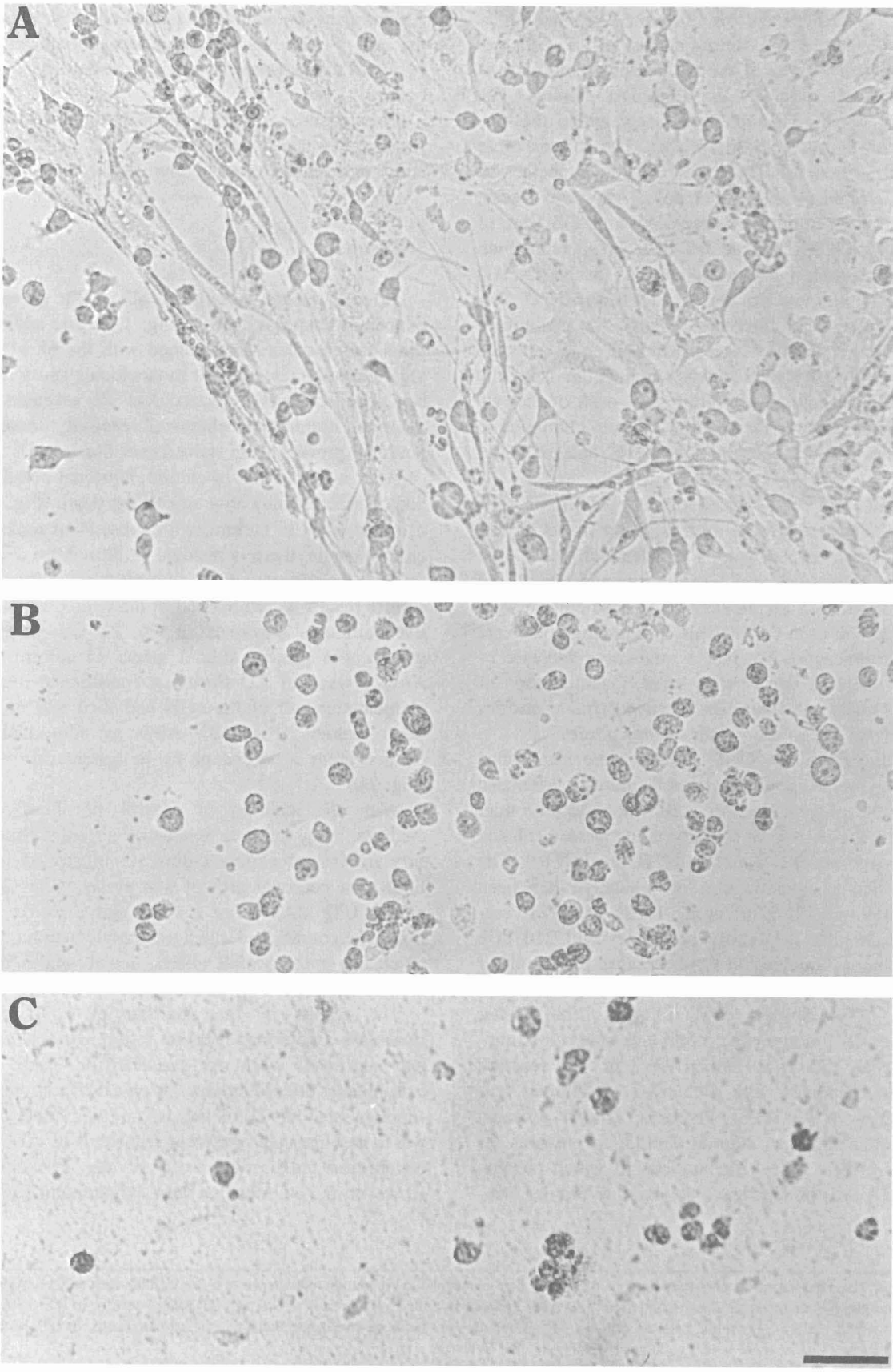
After a third week in culture, neuronal cellular profiles had contracted, indicative of cellular death (Fig. 1B). After a fourth week in culture, with subsequent replacement of culture media, there is residual cellular debri (Fig. 1C).

With the addition of AD fibroblast-conditioned medium, similar results were obtained in many cultures (representative culture is illustrated in Fig. 2). Exuberant neuronal growth was present after 2 weeks in culture (Fig. 2A). After 1 week of AD fibroblast-conditioned medium supplementation, all of the cells had died and only cellular debri remains (Fig. 2B). After an additional week in culture, there is no change in the appearance of the debri (Fig. 2C).

With the addition of control fibroblast-conditioned medium, many cultures revealed a different pattern of cell survival (representative culture is illustrated in Fig. 3). Exuberant neuronal growth was present after 2 weeks in culture (Fig. 3A). After 1 week and 2 weeks of control fibroblast-conditioned medium supplementation, there is no change in the overall cellular density and in the appearance of the cells (Fig. 2B and C).

The results of the addition of AD and control fibroblast-conditioned medium to the neuronal cultures for one additional week are presented in Table 1. In the cultures that did not receive fibroblast-conditioned medium supplementation, 11 of the 16 cultures died. This result was not statistically different from that of AD fibroblast-conditioned media where 10 of the 19 cultures died. However, the cultures that were supplemented with control

Fig. 1. (see page 26). Phase-contrast photomicrographs of murine E16 telencephalic vesicle neurons grown in tissue culture with nonconditioned medium. Each photomicrograph is of the same microscopic field. (A) After 2 weeks in culture, (B) after 3 weeks, and (C) after 4 weeks. In (A) there is exuberant neuronal growth with multiple pyramidal cells evident. In (B) all cellular profiles have contracted indicative of cellular death. In (C), with subsequent replacement of culture media, there is less cellular debris than in (B). Bar = 50 μm.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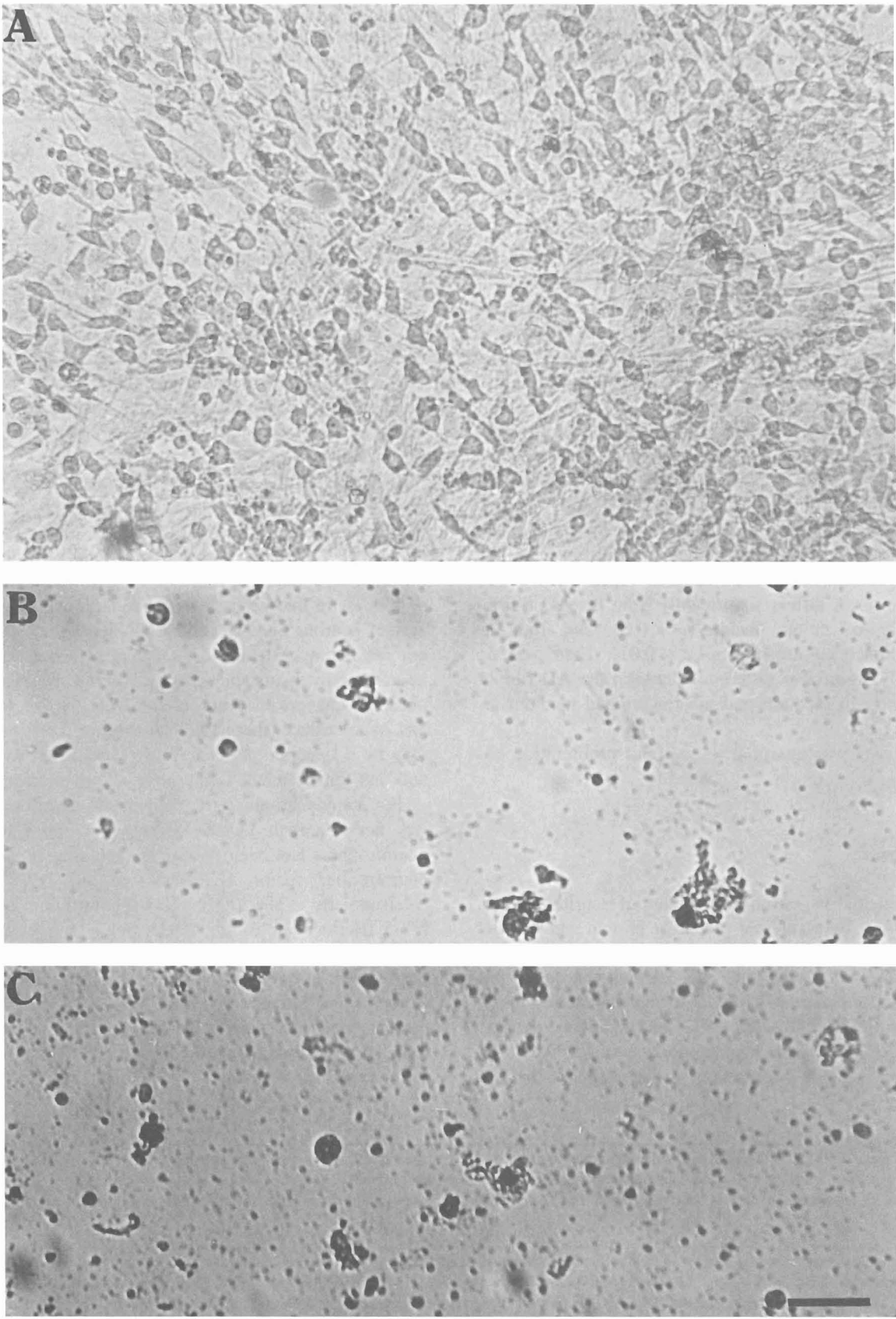
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Table 1
Effects of control and AD fibroblast-conditioned culture medium supplementation on murine CNS neurons after 1 week of supplementation (3 weeks in culture). There is less neuronal death with the control fibroblast-conditioned media as compared to AD ($p < 0.025$) or to no supplementation ($p < 0.005$). There is no statistically significant difference between the AD fibroblast-conditioned and nonconditioned culture results.

	No change	Died
AD	9	10
Controls	16	2
Nonconditioned	5	11

Table 2
Effects of control and AD fibroblast-conditioned culture medium supplementation on murine CNS neurons after 2 weeks of supplementation (4 weeks in culture). There is less neuronal death with the control fibroblast-conditioned media as compared to AD ($p < 0.05$) or with no supplementation ($p < 0.01$). There is no statistically significant difference between the AD fibroblast-conditioned and nonconditioned culture results.

	No change	Died
AD	3	16
Controls	8	10
Nonconditioned	0	16

fibroblast-conditioned medium survived significantly better: only 2 of the 18 cultures died. This difference is statistically significant in comparison with the AD fibroblast-conditioned cultures ($p < 0.025$) and with the nonconditioned cultures ($p < 0.005$). There was no statistically significant difference between AD fibroblast-conditioned medium and nonconditioned medium.

The results after 2 weeks of supplementation are presented in Table 2. In the cultures that had not received conditioned medium supplementation, all 16 died. This result did not differ significantly from that of the AD fibroblast-conditioned cultures where 16 of the 19 died. Again, there was a statistically significant difference in the control fibroblast-conditioned cultures where 10 of the 18 died. This result differs significantly from the AD fibroblast-conditioned culture results ($p < 0.05$) and from the nonconditioned culture results ($p < 0.01$). There was no statistically significant difference between the AD fibroblast-conditioned medium and nonconditioned medium results.

In no case was neuronal growth and proliferation observed.

4. Discussion

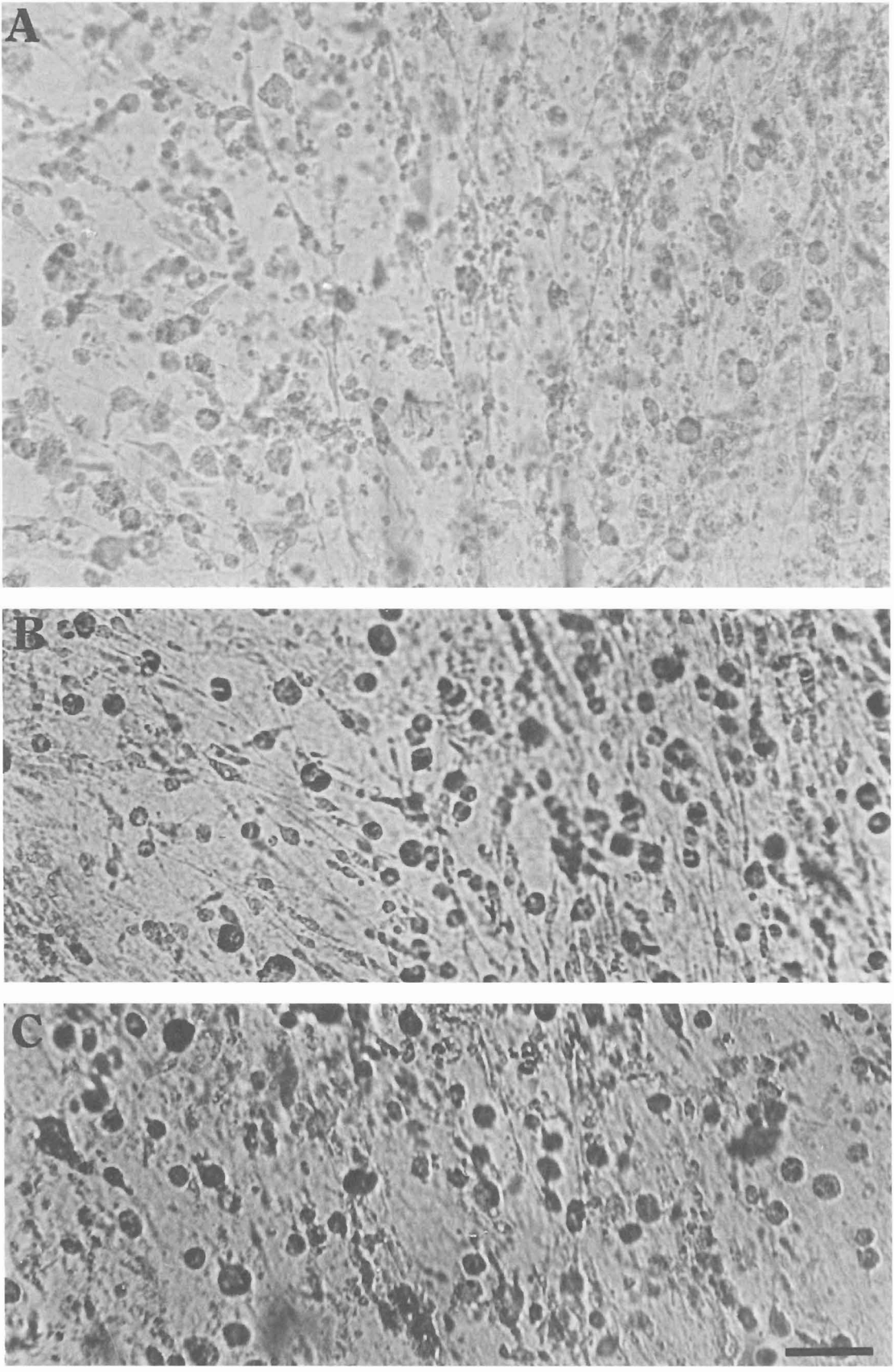
Beta-amyloid is a major component of neuritic plaques found in AD. Beta-amyloid precursor protein and its mRNAs are expressed in cultured AD skin fibroblasts (Dooley et al., 1992). Beta-amyloid has been shown to inhibit sprouting and survival of cultured chick and rat sympathetic neurons (Roher et al., 1991) and to induce neuritic dystrophy in cultured rat hippocampal neurons (Pike et al., 1992). One possible explanation of our results is that the AD-conditioned medium contained beta-amyloid and that what we observed is a result of toxicity to cultured neurons. This explanation is unlikely because the AD results did not differ significantly from the nonconditioned medium results. If the AD fibroblast-conditioned medium had contained toxic properties, there should have been much more neuronal death with this medium than with the nonconditioned medium, an event which we did not observe (Tables 1 and 2).

Our results suggest the absence of a CNS neuronal growth-sustaining factor in AD which is present in controls. The techniques that we used were similar to those of Kessler (1987) who showed a deficiency of a cholinergic differentiating factor in AD. Our observations extend those of Kessler in that he used peripheral sympathetic nervous system neurons and we used CNS neurons. Furthermore, our results suggest that in AD there is a deficiency of a neuronal growth-sustaining factor whereas Kessler had not observed changes in neuronal survival. As Kessler (1987) had hypothesized, these findings suggest that in AD there may be a systemic deficiency of neuronal growth factors and that this deficiency may be a causative factor in AD.

For many years the only recognized neurotrophic factor was nerve growth factor. Recently an entire family of neurotrophins has been described including brain derived neurotrophic factor (Leibrock et al., 1986), NT-3 (Maisonpierre et al., 1990), NT-4 (Hallbrook et al., 1991), NT-5 (Berkemeier et al., 1991) and ciliary neurotrophic factor (Adler et al., 1979). Alpha/beta interferon has also been shown to have neuronal growth-promoting properties (Plioplys and Massimini, 1992). What relation, if any, this possible neuronal growth-sustaining factor in control fibroblast-conditioned medium has to this family of neurotrophins is not known.

Fig. 2. (see page 27). Phase-contrast photomicrographs of murine E16 telencephalic vesicle neurons grown in tissue culture medium to which AD fibroblast-conditioned medium was added. Each photomicrograph is of the same microscopic field. (A) After 2 weeks in culture, (B) after 3 weeks, and (C) after 4 weeks. In (A) there is exuberant neuronal growth. In (B) all cellular profiles have contracted indicative of cellular death. In (C) only cellular debris persists. Bar = 50 μm.

Fig. 3. Phase-contrast photomicrographs of murine E16 telencephalic vesicle neurons grown in tissue culture medium to which control fibroblast-conditioned medium was added. Each photomicrograph is of the same microscopic field. (A) After 2 weeks in culture, (B) after 3 weeks, and (C) after 4 weeks. In (A) there is exuberant neuronal growth. In (B) and (C) there is no change in the overall cellular density and in the appearance of the cells. Bar = 50 μm.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It is conceivable that our results may be due to a generalized deficiency of protein secretion from AD fibroblasts. This possibility needs to be investigated further. Likewise a possible relationship of this result to the expression of ApoE4 needs to be investigated. It should be noted that our observations are based on the results from only two AD patients and two controls. Further investigation with larger numbers of patients and controls is warranted.

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