Effects of Serum From Patients With Type 1 Diabetes on Primary Cerebellar Granule Cells

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Type 1 diabetes is an autoimmune disease of unknown etiology. Our previous work has shown that a factor present in serum from type 1 diabetic patients causes increased Ca\(^{2+}\) channel activity and apoptotic DNA fragmentation in pancreatic \(\beta\)-cells. Here we examined the effects of type 1 diabetic serum on primary cerebellar granule cells (CGCs). In CGCs, exposure to type 1 diabetic serum did not cause increased apoptosis or changes in Ca\(^{2+}\) channel activity. However, patient serum did cause modulation of Ca\(^{2+}\) signals in a cell type with triangular soma that exhibited low voltage-gated Ca\(^{2+}\) currents. This cell was present primarily in cultures exposed to type 1 diabetic serum. The presence of low voltage-gated Ca\(^{2+}\) currents and long neuronal dendrites indicated that this unique cell was of neuronal origin and not of glial origin.

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Received for publication 6 July 2000 and accepted 15 August 2000.

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This article is based on a presentation at a symposium. The symposium and the publication of this article were made possible by an unrestricted educational grant from Les Laboratoires Servier.

AraC, 1-β-D-arabinofuranosylcytosine; [Ca\(^{2+}\)], cytosolic free Ca\(^{2+}\) concentration; CGC, cerebellar granule cell; FACS, fluorescence-activated cell sorting; FIGE, field inversion gel electrophoresis; PBS, phosphate-buffered saline; PI, propidium iodide.

Diabetes 50 (Suppl. 1):S77–S81, 2001

RESEARCH DESIGN AND METHODS

Preparation of sera. Sera from newly diagnosed type 1 diabetic patients and from control healthy subjects were collected, sterile-processed, and stored at −20°C until use. The sera were heat-inactivated by incubation at 56°C for 30 min and tested for effects of K\(^+\) depolarization on [Ca\(^{2+}\)]\(_i\) \(\beta\)-cells from ob/ob mice cultured overnight in the presence of 10% diabetic or normal serum. In primary \(\beta\)-cells, upon depolarization, all diabetic sera used induced a higher increase in [Ca\(^{2+}\)]\(_i\), compared with that caused by serum from healthy donors.

Isolation of cerebellar granule cells from rat pups. CGCs were isolated from 8-day-old rat pups as described previously (4). Briefly, the cerebellum was carefully removed, meninges and blood vessels were removed under a dissecting microscope, and the tissue was sliced and then trypsinized for 15 min with gentle shaking at 37°C. Trypsinization was halted with a stop solution, cells were centrifuged at 4,000 rpm for 1 min and resuspended in a small amount of medium, and an 18-gauge needle was used to triturate the cells five times. Cells were then acquired by centrifugation at 500 rpm for 15 min, counted, and plated at a density of 2.5 × 10⁶/ml. After 48 h in culture, 10 µmol/l 1-β-D-arabinofuranosylcytosine (AraC) was added to the cultures to avoid proliferation of glial cells or fibroblasts. In 8-day-old animals, other types of cerebellar neurons have reached the differentiating (postmitotic) stage of development, whereas granule cells are capable of undergoing mitosis. Glial cells are still at the mitotic phase but can be eliminated during treatment with AraC. Thus, the advantage of this type of culture is that it consists of almost 90% granule cells, which are characterized as glutamatergic neurons.

Qualitative analysis of DNA fragmentation. Formation of high-molecular-weight DNA fragments was determined by using field inversion gel electrophoresis (FIGE), as previously described (5). Briefly, after treatment, 1 × 10⁶ cells were resuspended in a solution containing 150 mmol/l NaCl, 2 mmol/l KH₂PO₄, pH 6.8, 1 mmol/l EGTA, and 5 mmol/l MgCl₂. An equal volume of 1% sodium lauroylsarcosine, and 200 µg/ml proteinase K and incubated for 24 h at 50°C with continuous agitation. The plugs were rinsed three times for periods of 2 h at 4°C in 10 mmol/l Tris-HCl, pH 8.0, and 1 mmol/l EDTA (TE buffer). Subsequently, they were stored until use at 4°C in 50 mmol/l EDTA, pH 8.0. Plugs were introduced into the 1% agarose gel, and FIGE was carried out using a horizontal gel chamber, a power supply, and a Switchback pulse controller (Hoefer Scientific Instruments, San Francisco, CA). Electrophoresis was run at 170 V in 0.5 × TBE (45 mmol/l Tris, 1.25 mmol/l EDTA, and 45 mmol/l boric acid, pH 8.0) at 12°C, with the ramping rate changing from 0.8 to 30 s over a 24-h period, applying a forward to reverse ratio of 3:1. DNA size calibration was performed using two sets of pulse markers, chromosomes from Saccharomyces cerevisiae (225–2,200 kbp), and a mixture of ADNA HindIII fragments, ADNA, and ADNA concatamers (0.1–200 kbp) purchased from Sigma (St. Louis, MO). DNA was stained with ethidium bromide, visualized using a 305-nm ultraviolet light source, and photographed using Polaroid 665 positive/negative film. Formation of oligonucleosomal DNA fragments was visualized by using conventional agarose gel electrophoresis. Cells were resuspended in 15 µl ultra-pure water and 6 µl of 50 mg/ml RNase A. After incubation for 20 min at room temperature, the loading buffer was added, and DNA was resolved on an 1.8% agarose gel containing 1 mg/ml proteinase K in 1 × TBE at 20 V overnight, followed by 1 h at 90 V. Resolved gel was incubated for 3 h in TBE buffer containing RNase A, stained with ethidium bromide, and photographed using Polaroid 665 positive/negative film.

Quantitation of DNA fragmentation. Quantification of apoptosis by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis was performed as described previously (6). Following incubation with various sera, cells were pelleted by centrifugation and resuspended in phos-
RESULTS

Incubation of granulocytes with normal or type 1 diabetic serum causes a distinct growth pattern. CGCs were isolated and immediately incubated in media containing 10% fetal calf serum, 10% normal human serum, or 10% type 1 diabetic serum. Major differences among CGCs incubated in the various sera were already apparent 3 days after isolation. The granular cells cultured in fetal calf serum adhered to the surface of the poly-L-lysine–treated coverslips much better than CGCs in diabetic and normal human serum, and a homogeneous population of typical granular cells was visible in the medium containing fetal calf serum (data not shown). In contrast, granular cells cultured in either normal human or type 1 diabetic serum were clustered and developed a network of dendrites (data not shown). However, no obvious differences between CGCs exposed to normal human or type 1 diabetic serum were apparent.

No apparent increase in apoptosis in neurons incubated with type 1 diabetic serum. Chromatin fragmentation was measured using three independent methods. Formation of high-molecular-weight DNA fragments (50 kb) was quantitatively assessed using pulse field gel electrophoresis (Fig. 1B). Oligonucleosomal DNA fragments were also visualized electrophoretically in CGCs incubated in the presence of calf serum, normal human serum, or type 1 diabetic serum for 3 days (Fig. 1A). To quantitate cells that were undergoing DNA fragmentation, we permeabilized and stained cells with propidium iodide and measured the percentage showing subdiploid amounts of DNA. This method yielded data that matched the DNA laddering results (Fig. 1C). All three measures of apoptotic DNA fragmentation showed no significant increase in apoptosis in CGCs exposed to type 1 diabetic serum compared with normal human or fetal calf serum.

Effects of type 1 diabetic serum on voltage-gated Ca2+ currents. DNA fragmentation is associated with increased activity of L-type Ca2+ channels (1). In the present study, we wondered whether exposure to type 1 diabetic serum could cause modulation of Ca2+ signals in CGCs in the absence of apoptotic DNA fragmentation. To address this question, we evaluated current-voltage relationships in CGCs incubated with fetal calf serum, normal human serum, and type 1 diabetic serum for 3 or 6 days. As shown in Fig. 2A and D, collectively there was no marked difference in Ca2+ current traces generated by a set of depolarizing voltage pulses (100 ms) between −70 and 40 mV in 10-mV increments from...
a holding potential of –80 mV. Compiled data showed that treatment with diabetic serum did not significantly alter Ca^{2+} current density in comparison with treatments with normal human and fetal calf serum (Fig. 2A and D).

However, two types of cells were present in cultures exposed to type 1 diabetic serum. The majority of cells were CGCs (Fig. 3C), but a few cells exhibited irregular triangular soma, in contrast to the more regular rounded soma seen in
Fig. 3A or B, with distinct processes (Fig. 3C). This type of cell was rarely found in CGCs exposed to normal human serum (Fig. 3B) and never found in cultures incubated with calf serum. After 6 days, the morphology of this type of cell worsened, and punctate structures were seen in the cytoplasm.

Interestingly, this distinct neuron displayed low voltage-gated Ca\(^{2+}\) currents (Figs. 2B, i and ii) that were absent in typical CGCs (Fig. 2A, v). The presence of a low voltage-gated Ca\(^{2+}\) current indicated that this cell can be functionally defined as a neuron. To better define this distinct neuron, we attempted immunostaining with antibodies directed toward neuron-specific enolase and glial fibrillary acidic protein. The cell in question did not stain positively for either of these markers (data not shown), suggesting that at day 3 after isolation, these markers may not be expressed (9).

**DISCUSSION**

[Ca\(^{2+}\)]\(_i\) is a pleotropic second messenger, and increases in [Ca\(^{2+}\)]\(_i\), can promote a variety of cellular responses, ranging from proliferation, via activation of Ca\(^{2+}\)-dependent transcription factors, to apoptosis. Our previous work has shown that exposure to serum from type 1 diabetic patients causes pancreatic \(\beta\)-cells to undergo increased Ca\(^{2+}\) channel activity and apoptotic DNA fragmentation (1). In the present study, we attempted to transpose that study onto a nonpancreatic cell. We were motivated to select a neuronal cell model because diabetic complications often involve neuropathies. We chose primary rat CGCs because these cultures are one of the best characterized models of normal neuronal cells. Human neuronal cell lines are often derived from neuroblastomas and are therefore not representative of a normal population of neurons.

Exposure of CGCs to type 1 diabetic serum promoted the growth of a unique cell, functional as a neuron, as indicated by the presence of low voltage-gated Ca\(^{2+}\) currents and long neuronal dendrites. However, its morphology was distinctive; instead of a rounded cell body with few dendrites (as seen in Fig. 3B), it had irregular triangular soma from which several dendrites stemmed (Fig. 3C). These findings suggest that a component present in type 1 diabetic serum can promote growth of this unusual neuronal cell. Attempts to better identify this cell were hampered by the fact that the morphology and capacity for Ca\(^{2+}\) currents began to worsen after day 3 of isolation. Normally, CGC cultures must differentiate for 1 full week in culture before experiments are conducted (4). This may explain why staining of this unique cell with an antibody directed toward neuron-specific enolase was negative (data not shown).

Our previous data suggest that Ca\(^{2+}\)-mediated apoptosis is a key factor in pancreatic \(\beta\)-cell death. However, in the CGC model, type 1 diabetic serum did not affect either apoptosis or Ca\(^{2+}\) channel activity. An obvious problem with our model is that serum from human type 1 diabetic patients was tested on rat CGCs. Exposure of rat CGCs to serum from rats with a type 1 diabetic phenotype could have represented a better alternative.

**ACKNOWLEDGMENTS**

J.C. is the recipient of a fellowship for visiting scientists from the Wenner-Gren Foundation. This work was supported by grants from the Swedish Medical Research Council (03X-2471, 72X-00890, 72X-00891, 72XS-12708, and 72X-00034), the
Swedish Diabetes Association, the Nordic Insulin Foundation Committee, the Fredrik and Ingrid Thurings Foundation, Funds of Karolinska Institutet, the Berth von Kantzows Foundation, the Novo Nordisk Foundation, the Swedish Society for Medical Research, and the Swedish Cancer Society (3829-B98-03XAC).

The authors gratefully acknowledge the assistance and advice of Eva Ahlbom and Sandra Ceccatelli.

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