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Short Report

Purified secretory glycoproteins of the bovine subcommissural organ influence neuronal differentiation *in vitro*

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ABSTRACT The subcommissural organ-spondin (SCO-spondin), a glycoprotein belonging to the thrombospondin family, is secreted by specialyzed cells located at the floor and roof plates during the ontogenetic development of the central nervous system, and by the subcommissural organ (SCO) in adulthood. The intracellular secretion of the bovine SCO was purified by immunoaffinity chromatography with monoclonal antibodies raised against the Reissner´s fiber (RF), a fibrous structure present at the ventricular cavities and that results from the polymerization of the SCO-spondin apically released by the SCO secretory cells. The purified secretion was used as a SCO-spondin source and assayed on cultures of the rat neuroblastoma B104 cell line. High doses of SCO secretory glucoproteins inhibited the proliferation of cultured neuroblasts, while lower concentrations stimulated neurite outgrowth and cellular aggregation.

During ontogenesis, floor plate and roof plate neuropepithelial cells produce morphogenetic molecules involved in neuronal differentiation, cellular adhesion and axonal guidance, such as F-spondins and mindins mainly secreted by the floor plate cells and belonging to the thrombospondin superfamily (Klar *et al.,* 1992; Higashijima *et al.,* 1997). Recently, a novel protein of this family, the SCO-spondin, has been sequenced (Gobron *et al.,* 2000). This protein is synthesised by specialized cells in the embryonic floor and roof plates and it has been proposed that it is released apically into the ventricular lumen. During ontogenesis, floor plate cells cease to express SCO-spondin, while a discrete area of the rostral roof plate differentiates into an ependymal gland, the SCO. This gland secretes into the ventricle glycoproteins of high molecular weigh, most of them condense to form the Reissner´s fiber (RF), which extends along the cerebral ventricles and the central canal of the spinal cord (Rodríguez *et al.,* 1988). Part of the ventricular secretion of the SCO has been proposed to remain soluble in the cerebrospinal fluid (Rodríguez *et al.,* 1993). The SCO-spondin exhibits numerous thrombospondin type I repeats (TSR) (Gobron *et al.,* 2000), thus suggesting a putative role of this protein in central nervous system development. Monnerie *et al.* (1997, 1998), showed that glycoproteins solubilized from RF and synthetic peptides containing the TSR sequence of SCO-spondin increased neuronal survival in primary cultures of chick embryo cortical and spinal cells. Likewise, El Bitar *et al.* (1999) showed improved neurite outgrowth and neuronal aggregation in cultured neuroblasmoma B104 cell line in response to RF material.

The aim of the present study was to assay the putative biological activity of the intracellular secretory glycoproteins of the bovine SCO on neuroblastoma B104 cell line. Intracellular secretion was purified from native SCO extracts by affinity chromatography with a monoclonal antibody raised to the bovine RF.

Extracts containing 400 adult bovine SCO were loaded onto an inmunoaffinity columm with 1 mg a monoclonal antibody anti-RF (3E6, Pérez *et al.,* 1996) bound to 1 ml of sepharose.

The eluted fraction was analyzed by the following methods: i) a specific sandwich ELISA with a polyclonal and monoclonal antibodies (Estivill-Torrús *et al.,* 1998) against bovine RF demonstrated that more than 90 % of total protein were secretory; ii) with PAGE-SDS and silver nitrate staining a pattern consisting in three intense bands of 540, 450 and 320 kDa with a few minor bands of lower molecular weight was observed (Fig. 1, left); iii) Western blotting with a polyclonal antibody against RF revealed the three molecular weight bands (Fig. 1, right).

This preparation of purified intracellular secretory proteins of the bovine SCO was assayed in rat B 104 neuroblastoma cells. B 104 cells were plated in 48-wells plates coated with poly-L-Lysine, at a density of 625 cell/cm2. Cells were cultured in Dulbecco modified Eagle medium (DMEM) suplemented with 10 % of fetal calf serum and 5 % of horse serum. Four to 6 hours after seeding, the complete medium was replaced by serum-free medium containing 0 (control), 5 or 50 μg/ml of purified SCO glycoproteins. The medium was not changed throughout the experiment. After four days, cultures were analyzed under an inverted microscope.

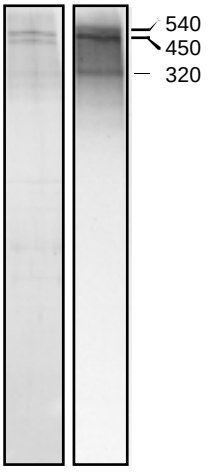

Fig. 1. Electrophoretic pattern of the intracellular secretion of the bovine subcommissural organ (SCO) purified by immunoaffinity chromatography. (Left lane), *silver nitrate staining;* **(right lane)**, *immunoblotting using a polyclonal antiserum against bovine Reissner fiber (RF). Molecular weights of the three secretory polypeptides in kDa.*

In control cultures (Fig. 2a) cell proliferation was patent, with most of the neurons displaying a bipolar morphology with two short neurite extensions. Cells remained

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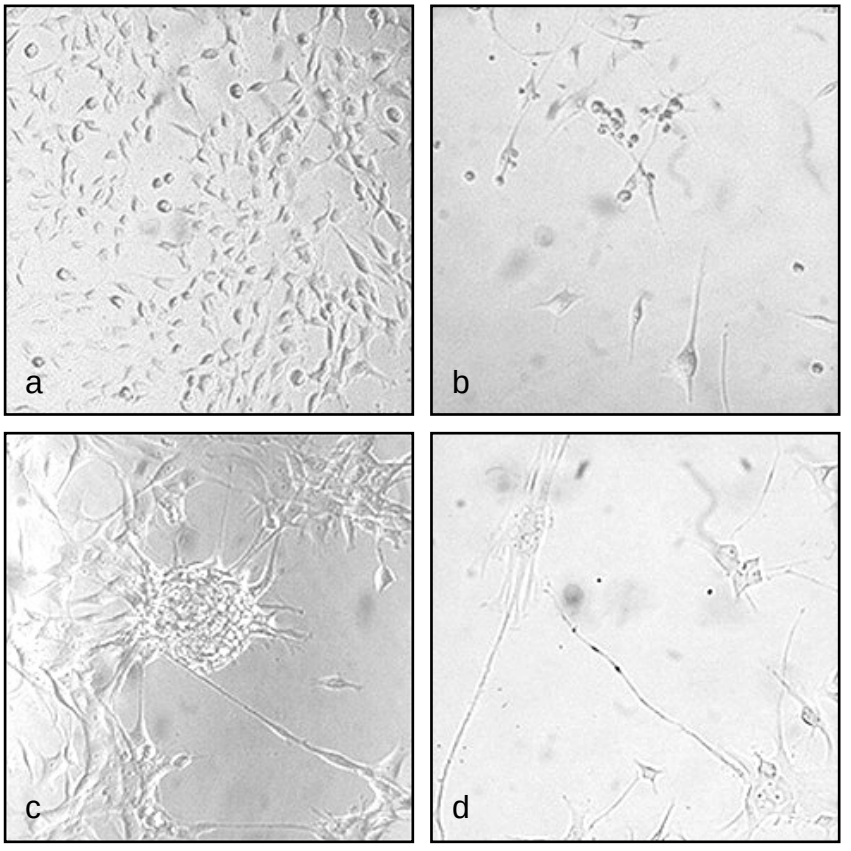

Fig. 2. Influence of subcommissural organ (SCO) purified secretion on neuronal aggregation and neurite outgrowth of B104 cells plated at low density. *Phase-contrast micrographs (140x) were taken at day 4 for cells cultured in serum-free medium in the absence (a) or presence (b,c,d) of purified SCO secretion at 50 (b) and 5 (c,d) μg/ml.*

evenly distributed and aggregates were rarely observed. In wells containing 50 μg/ml of purified SCO-secretion (Fig. 2b) neuronal proliferation was strongly inhibited with only scarce isolated cells being observed. Cellular death was abundant as demonstrated by MTT assay. By contrast, in the presence of 5 μg/ml of SCO-secretion B 104 cell showed a strong tendency to form compact aggregates with abundant neurites bundles establishing contacts between them (Fig. 2c). Furthermore, the cells that remained isolated developed long neurites that frequently contacted a cellular aggregate or other isolated neuron (Fig. 2 c,d).

The present results show that bovine SCO-purified glycoproteins enhace neuronal aggregation and neurite outgrouth in cultures of B104 neuroblastoma cells. Similar effects have been reported by aplication of bovine RF-glycoproteins to the same cellular line (El Bitar *et al.,* 1999). These results support the possibility that SCO-spondin, like other proteins of the thrombospondin superfamily, could participate in the central nervous system development.

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