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Short Report

Collagen as Adherent Substratum and Inducer of Dorsal Root Ganglia Outgrowth

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ABSTRACT

Neurite outgrowth from dorsal root ganglion (DRG) explants is a method of evaluating neurotrophic activity of growth factors. When complete medium containing collagen was supplemented with nerve growth factor (NGF) DRG outgrowth was observed after 18 h. In the absence of NGF and in the presence of collagen, the DRG outgrowth took place after 72 h. In wells not supplemented with collagen gel in substratum, no DRG outgrowth was observed. Partially, DRG differentiation was observed in the presence of NGF. In the absence of NGF and collagen, there was no DRG outgrowth detected. It seems that, in some circumstances, cells degenerated by DRG may be an indication of an apoptosis phenomenon. Therefore, we suggested that collagen as a substratum is more effective than NGF. *Iran. Biomed. J. 8 (2): 101-105, 2004*

Keywords: Collagen, Adherent substratum, Dorsal root ganglia, Culture, Neurite

INTRODUCTION

Neurite outgrowth from primary culture of dorsal root ganglion (DRG) explants is a commonly used method for evaluating neurotrophic effect of growth factors and growth factor mimetics [1]. Historically, nerve growth factor (NGF) was identified by promoting neurite from primary cultures of chick explants. Although, neurite outgrowth from DRG explants is a robust means of identifying neurotrophic activity [2].

Cell adhesion is an important factor in cell culture. Cell adhesion molecules (CAM) such as laminin, collagen, fibronectin and vitronectin are cell membrane macromolecules important in controlling cell-to-cell adhesion during development by influencing neurite outgrowth, neural migration, adhesion, synaptogenesis, intracellular signaling and apoptosis [2-6]. Integrin is an important adhesion receptor that causes the binding of extracellular matrix to cellular cytoskeleton, and participates in intercellular bindings [7, 8]. Collagen as an adhesion molecule has been used especially for nerve cell culture. This protein has Arg-Gly-Asp (RGD) motifs and Lys that can lead to the binding of the adhesion receptors to the cell surface, therefore cause the induction of some intracellular signals for cell growth and differentiation [8, 9]. In such studies, collagen was used as an essential factor in both growth and differentiation of DRG. The aim of this study was to show the essential roles of collagen compared to NGF in differentiation and neurite outgrowth of DRG.

MATERIALS AND METHODS

Preparation of collagen solution. Collagen solution was extracted and prepared from the rat tail (200 g) as previously reported by Michalopolaus and Pitot [10]. Briefly, rat tail collagen fibers (1 g) was sterilized in alcohol overnight, then dissolved in 300 ml of acetic acid (1:1000 dilution in sterile distilled water) and

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stirred at 4°C for 48 h. Subsequently, the solution was left to stand without agitation for 24 h allowing the undissolved fibers to sediment. The clear solution is gently poured off into a sterile container.

Preparation of collagen gel. The 8/2 ratio of collagen and DMEM medium (Bahar-Affshan, Tehran, Iran) was used. This solution is yellow and acidic, 2-3 drops of sterile 1N NaOH was added until the solution became reddish as an indication of neutral medium [11].

Extraction of 7s NGF. The 7s NGF was extracted from 100 submaxillary gland of mature male mice according to the previously reported method [12]. Briefly, submaxillary gland was used to isolate the 7s NGF. The method comprises of gel filtration on Sephadex G-100 chromatography Sephadex on DEAE cellulose and gel filtration on Sephadex G-150.

Assay of 7s NGF protein. The 7s NGF content was determined according to Lowry *et al.* assay [13]. In this experiment, BSA, at concentrations of 0, 20, 40, 60, 80 μg was used for standard curve determination. The extracted NGF gave a concentration of 5.7 mg/ml. Bioassay of NGF was done using E8 chick and optimized concentration of 100 ng/ml.

Isolation of DRG from E8 chicken and its application in cultivation. Chick embryos were killed by decapitation after 8 days of incubation. The skin was stripped from the dorsal surface of the body, and the embryo eviscerated. Ganglia were removed from the abdominal region of the embryo and put into Hank′s buffer under stereomicroscopic conditions. In order to construct the collagen gel, 96 multiwell plates (Nunc) were used. Collagen gel (50 μl) was loaded in each well and incubated at 37°C, 5% CO_2 and 95% air for 30 min. Afterwards, 200 μl of DMEM + 10% FCS containing separate dose of the following doses of NGF 0, 10, 25, 50, 100, 200, and 300 ng/ml was added to each well. Aganglia was added to each separate well and incubated under the same mentioned conditions. Two wells were considered for each concentration. In order to study the role of collagen in the DRG outgrowth, control experiment was simultaneously carried out in the absence of collagen in the substratum.

RESULTS

Since neurite outgrowth from primary culture of DRG explant is a commonly used method for evaluating neurotrophic effect of growth factors, DRG outgrowth was observed 18-24 h of culturing in the presence of 100, 200, and 300 ng/ml of NGF. However, after 48-72 h, DRG outgrowth was observed at 10, 25 and 50 ng/ml of NGF. No outgrowth occurred at the same concentrations of NGF in wells lacked collagen in the bottom.

In the presence of collagen substratum but absence of NGF, DRG outgrowth was started after 2-3 days and completed one week later. On the other hand, even the application of six different doses of NGF in the absence of collagen no DRG outgrowth was induced (Fig. 1). However, the absence of both collagen and NGF caused no DRG outgrowth, but cell segregation and signals of apoptosis were observed (Fig. 2).

DISCUSSION

Extracellular matrix (ECM) molecules such as fibronectin, laminin, vitronectin and collagen, provide physical support, regulate ionic and nutritional homeostasis of surrounding cells, and possess ligands that interact with cell surface receptors initiating signaling events that guide a wide range of functions including cellular proliferation, motility, differentiation, neurotic outgrowth growth cone targeting, synapse stabilization and apoptosis [6]. Chen *et al.* [14] showed that engagement of integrins with ECM is not only required, but also subsequent cell spreading appears to be important. Integrin-dependent adhesion to cell matrix is required for the survival of many cell types, while in the absence of cell matrix interactions, cells soon undergo apoptosis [8, 15, 16].

Several studies have focused their efforts on the effect of collagen as an ECM on outgrowth of DRG and different results were reported. Carboretto and Cochard [17] reported that in culture medium lacking NGF, DRG neurons caused nerve fibers extension only on laminin but not on fibronectin, collagen or polylysin. In contrast Rochat *et al*. [18] reported that after few days in cultures, DRG explants grown on collagen substrate showed a flattened shape consisting of a core surrounded by a crown of neuritis that was mixed up with different

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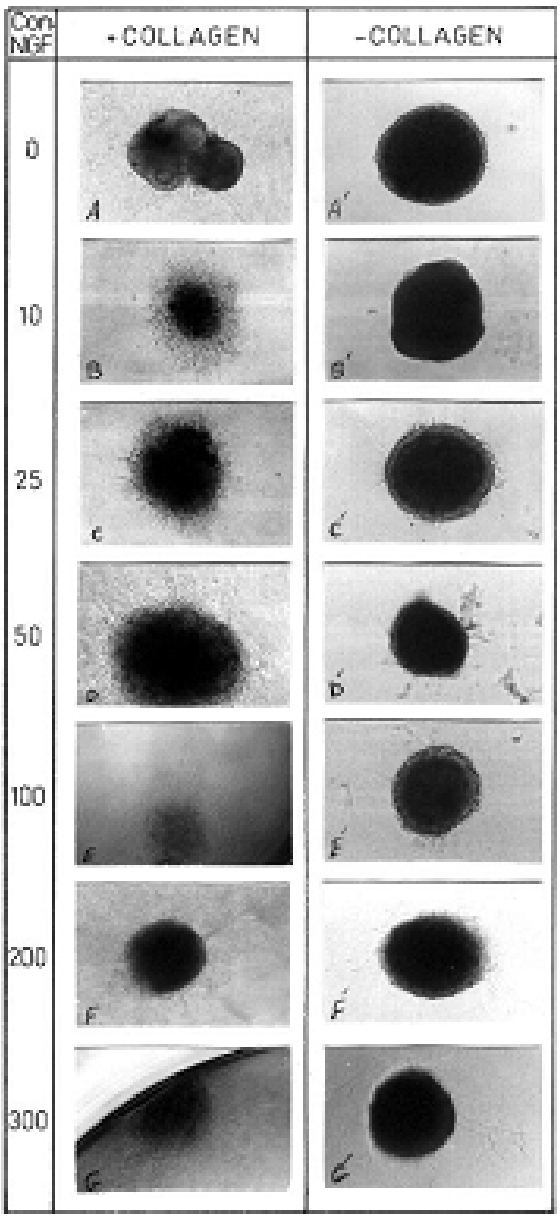


Fig. 1. Neurite outgrowth on collagen substratum. Dorsal root ganglia (DRG) from a 8-day chick embryo were cultured in a 96-multiwell plate coated with collagen and different concentration of NGF 0, 10, 25, 50, 100, 200 and 300 ng/ml. Ganglia were photographed after one day. The comparison of Figures: with and without collagen show no neurite outgrown in the absence of collagen, but with any concentrations of NGF. Magnification: 100 ×.

types of migrating cells. However, in the absence of NGF, Skaper *et al.* [19] compared collagen polyornitine (PORN) and PORN exposed to a polyornitine binding neurite promoting factor (PNPF-PORN) and observed a gradation of increasing neurite growth as follows: PNPF-PORN>PORN> collagen. In our studies, DRG outgrowth was noticed in the absence of NGF after few days on the collagen gel, while no such event was detected without collagen gel as an adhesive substratum. Collagen has RGD sequences that can interact with integrin on the surface of the nerve cells and can cause some signals in these cells [6]. So far, 23 types of integrins with 15α and 8β subunits have been identified [8]. Collagen type 4 as an important part of the structural component of basement membrane, can induce nervous outgrowth in both sympathetic nerves and DRG [8]. Venstrom and Richardt [20] showed that β1α1 and β8α1 integrins behaved as collagen receptors on neurons, but β1α2 acted the same on non-nervous cells. A recent documented role of integrin has been reported in signal transduction via focal adhesion kinase (FAK) [21-23]. In our studies, DRG outgrowth was not observed on the collagen after 18 h, but was observed after 2-3, days even without NGF.

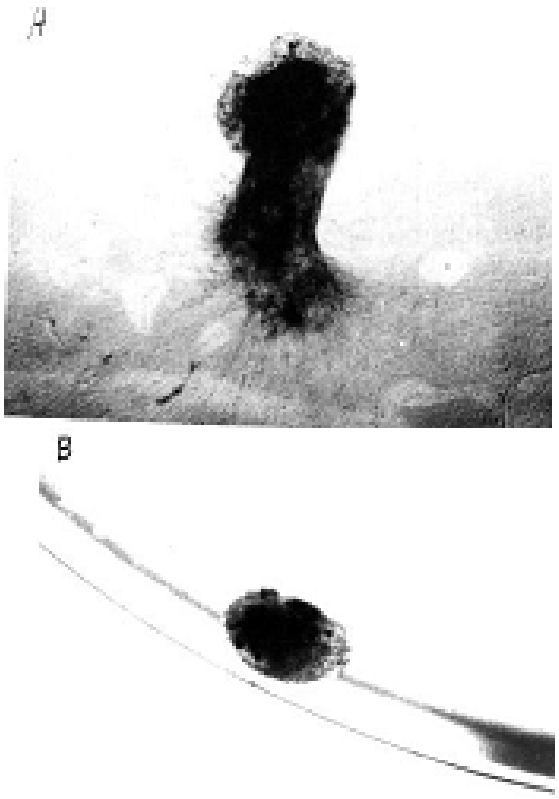


Fig. 2. (A), Neurite outgrowth in the presence of collagen in the substratum after two weeks without NGF; and **(B)**, shows DRG in the absence of NGF and collagen in the same time. Magnification: 100 ×.

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When NGF and collagen were present, NGF affected NGF receptors, and this in turn suggests that collagen gel can act as a footprint for growth of nervous fibers. However, in the absence of collagen, although NGF was applied at optimum concentrations, footprint, adhesion and signal transduction did not take place. DRG on collagen gel was alive and showed extreme outgrowth after one week. Whereas, in the absence of collagen gel, although NGF was present, no sign of live DRG was observed. It seems that separation of some cells from DRG may face apoptosis and subsequent death. It has been reported that NGF can cause integrin expression [24], however in the absence of collagen in DRG culture, although NGF was present, neurite outgrowth was not observed even in the presence of NFG. Recent data declared that ligands could regulate expression of integrins and cause control of cell adhesion and neurite outgrowth [25]. As a whole, it is possible that specific kinases (extracellular signal-regulated kinase) are activated when making contact with collagen, whereas in the absence of substratum, kinases such as amino terminal C-jun kinase or stress activated protein kinase are activated when they have a role in arresting cell cycle and apoptosis [26]. The role of microtubule in the control of signal transduction through adhesion process was recently reported [27].

Therefore, the role of collagen gel in DRG culture is essential and it is hard to regard it as an adherent substratum.

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