Endothelial Cell Mitogens Derived from Retina and Hypothalamus: Biochemical and Biological Similarities

PATRICIA A. D'AMORE* and MICHAEL KLAGSBRUN‡
*Departments of Pathology, ‡Biological Chemistry, and Surgery, Harvard Medical School and The Children's Hospital, Boston, Massachusetts 02115

ABSTRACT Bovine retina and hypothalamus contain anionic endothelial cell mitogens that display unusual affinities for the negatively charged glycosaminoglycan heparin. Both growth factor activities are acidic polypeptides (pI's of 5.0) as determined by isoelectric focusing and DEAE-affinity chromatography. In spite of their anionic nature, the factors bound to heparin-Sepharose columns with high affinity and could be eluted only at high salt concentrations (0.9–1.1 M NaCl). The affinity of the retina-derived growth factor (RDGF) for heparin permitted a 15,000-fold purification of the mitogen in two steps: heparin-affinity chromatography and size exclusion high-performance liquid chromatography. RDGF and the anionic hypothalamus-derived factor (aHDGF) exhibit three major biochemical similarities including isoelectric point, (pI's of 5.0), heparin affinity (elution at 0.9–1.1 M NaCl) and molecular weight (18,000). Additionally, the two factors display similar biological activities, stimulating the proliferation of capillary and human umbilical vein endothelial and 3T3 cells but not vascular smooth muscle cells. We suggest that RDGF and aHDGF are related if not identical growth factor molecules.

In a normal, adult tissue the endothelium exhibits a very low rate of turnover. The mitotic indices of the capillary endothelium have been documented to be 0.14% in the myocardium and as low as 0.01% in the retina (7). Yet, there are a number of conditions, both normal and pathological, in which endothelial cell proliferation increases. Inflammation, psoriasis, tumor growth, formation of the corpus luteum, and diabetic retinopathy all involve dramatic levels of new blood vessel growth. It is not clear in any of these cases what causes the stable growth controls that the endothelium appear to possess to go awry. Because proliferation is central to the formation of new blood vessels, factors that influence endothelial cell (EC)[1] proliferation may be involved.

EC mitogens have been identified in a wide variety of cells and tissues, including cartilage (17), tumors (21), platelets (4), brain (12, 15, 23), hypothalamus (18), and pituitary (9, 11). The relationship of these EC mitogens to one another (if any) is unclear. We (5, 10) and others (1) have previously demonstrated that extracts of bovine retina also contains a potent EC growth factor, retina-derived growth factor (RDGF). The recent finding by our group that an EC growth factor from chondrosarcoma exhibits a strong affinity for an anionic glycosaminoglycan heparin (21) prompted us to examine the binding of RDGF to immobilized heparin. We have now found that RDGF, which we know to be anionic, also displays a strong affinity for heparin. We used this unexpected affinity for heparin to facilitate the isolation of this growth factor from retina. We also investigated the nature of another EC mitogen that has been described to exist in hypothalamus (19). Here we show that this mitogen shares a number of biochemical characteristics and biological functions with RDGF and appears to be similar if not identical to the RDGF.

MATERIALS AND METHODS

Cell Culture and Proliferation Assays: Capillary endothelial cells. Bovine capillary EC from adrenal cortex were generously given to us by Dr. J. Folkman (The Children's Hospital, Boston, MA) and cultured as previously described (8). The growth factor activity of column fractions obtained throughout purification and characterization was assessed as follows. Capillary EC (passages 3–10) were plated at 10,000 per well in 2.1 cm^2 multiwell dishes (Nunc, Vanguard International, NJ) in Dulbecco's modified Eagle's medium with 10% calf serum. The cells were allowed to attach and recover from trypsinization overnight. The unattached cells and medium were aspirated and 0.5 ml of fresh medium was added together with the fraction to be tested in volumes up to 50 μl. 2 d later the cells were removed by trypsinization and cell number determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

BALB/c 3T3 Cells: Growth factor activity in column fractions was also monitored by measuring the stimulation of [methyl-^{3}H]thymidine incor-

[1] *Abbreviations used in this paper:* EC, endothelial cell; IEF, isoelectric focusing; aHDGF, anionic hypothalamus-derived growth factor; HPLC, high-performance liquid chromatography; RDGF, retina-derived growth factor

poration into 3T3 cells. This assay, which measures stimulation of DNA synthesis in quiescent monolayers of 3T3 cells, and procedures for maintaining the 3T3 cell cultures have been previously described (16). A unit of 3T3 cell stimulatory activity was defined as the amount of growth factor required to yield half-maximal [^{3}H]thymidine incorporation by the 3T3 cells.

Preparation of Retinal Extract: Retinas which were dissected from bovine eyes were incubated in Hank's balanced salt solution (one retina per milliliter) for 3 h at room temperature as previously described (10). The retinas were removed by centrifugation and the extract was subjected to the following separation techniques.

Hypothalamus-derived Growth Factor: A crude salt (0.15 M NaCl) extract of bovine hypothalami (Endothelial Mitogen) was purchased from Biomedical Technologies Inc., Cambridge, MA. The anionic hypothalamus-derived growth factor (aHDGF) used in this study was obtained by fractionation of the crude hypothalamic extract by IEF as described below.

Isoelectric Focusing: Isoelectric focusing (IEF) was conducted for both analytical and preparative purposes using an LKB 8100 vertical electrofocusing column. Ampholytes (LKB Instruments, Bromma, Sweden) from pH 3.5 to 10 were distributed on a linear sucrose density gradient (5–50% wt/vol). The crude retinal extract which had been dialyzed extensively against water was introduced at the time of gradient formation. Samples were focused at a constant voltage of 820 V for 24 h at 4°C. 55 2-ml fractions were then collected and their pHs were determined. The fractions were pooled in groups of three, dialyzed first against 1.0 M NaCl to facilitate the removal of the ampholytes, then exhaustively dialyzed against deionized, distilled water. Dialysis tubing with a molecular weight cut-off of 3,500 (Spectrum Medical Industries, CA) was used in all dialysis steps. The samples were then lyophilized and tested for their ability to stimulate capillary EC proliferation and 3T3 cell DNA synthesis.

Heparin-Sepharose Affinity Chromatography: Heparin-Sepharose affinity chromatography was conducted using a 7 × 1 cm column of heparin-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, at 4°C. The active fractions obtained from IEF of RDGF and aHDGF were dialyzed against the column equilibration buffer and applied to separate columns of heparin-Sepharose. After rinsing with equilibration buffer the growth factors were eluted with a gradient of 0.1 to 3.0 M NaCl in 0.01 M Tris-HCl at 40 ml/h. The fractions were then dialyzed as after the IEF column.

Size Exclusion Chromatography: Both the crude retinal extract and the peak of growth factor activity obtained from heparin-Sepharose chromatography were fractionated on a TSK 2000 size exclusion column (Varian, 8mm I.D. × 50 cm), which was equilibrated with 0.6 M NaCl, 0.02 M Tris-HCl, pH 7.0, using a Beckman model 334 HPLC system (Beckman Instruments, Palo Alto, CA). HPLC was conducted at room temperature at a flow rate of 0.5 ml/min. Fractions (0.8 ml) were collected, dialyzed as above, and tested for their ability to stimulate proliferation of capillary EC and DNA synthesis by 3T3 cells. Blue dextran (2 × 10^6), serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), and lysozyme (14,300) were used to calibrate the column.

RESULTS

Characterization of RDGF

We had reported previously that bovine retina can release under the extraction procedures described here a polypeptide factor that is mitogenic for fetal aortic and capillary EC as well as BALB/c 3T3 cells (5, 10). Analysis of this retinal extract by IEF revealed a single peak of growth factor activity that focused with a pI of 5.6 ± 0.3 and stimulated the proliferation of capillary EC as well as DNA synthesis by 3T3 cells (Fig. 1).

The growth factor activity obtained from IEF shown in Fig. 1 was applied to a column of heparin-Sepharose (Fig. 2). Whereas >90% of the applied protein eluted from the column in the void volume (protein profile not shown), most of the remaining protein was eluted from the heparin-Sepharose before 0.5 M NaCl. Less than 8% of the growth factor activity was associated with the void volume. The majority of the growth factor activity eluted from the column between 0.9 and 1.1 M NaCl as a single peak of activity that stimulated both the capillary endothelial and the 3T3 cells. Similar results were obtained when the crude extract was applied directly to

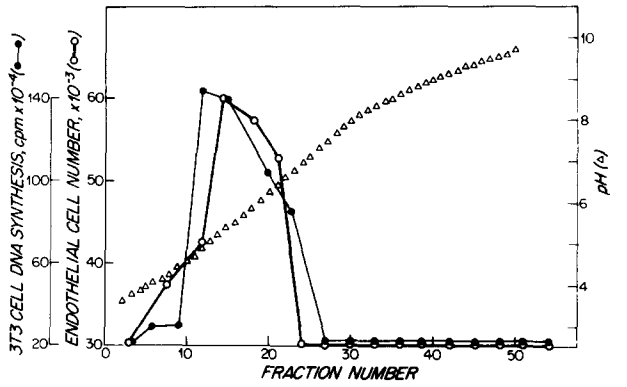

FIGURE 1 IEF profile of RDGF. Crude retinal extract (30 ml, 180 mg protein) was analyzed by IEF on a vertical electrofocusing column using ampholytes of pH 3.5–10 distributed on a linear sucrose density gradient. 55 2-ml fractions were collected and the pH (△) determined. The fractions were pooled in groups of three, dialyzed first against 1.0 M NaCl to facilitate the removal of the ampholytes and then against deionized, distilled water. The fractions were then lyophilized, dissolved in phosphate-buffered saline and tested for their growth factor activity. A representative column is shown in this figure. EC number (○) is the average of triplicate wells: 3T3 cells [^{3}H]thymidine incorporation (●) is the average of two wells.

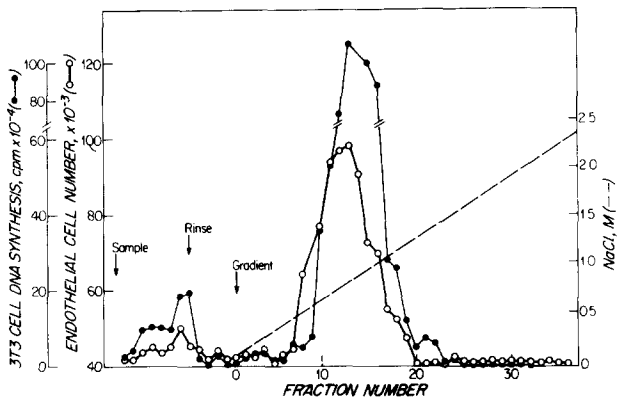

FIGURE 2 Heparin-Sepharose chromatography of RDGF obtained from IEF. The peak of growth factor activity obtained from IEF (fractions 12–22; Fig. 1) was dialyzed against 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, and applied to a column of heparin-Sepharose (1 × 7 cm) equilibrated in the same buffer. After a 30-ml rinse with the equilibration buffer the protein was eluted at 40 ml/h with a gradient of 0.1–3.0 M NaCl (200 ml). The fractions were dialyzed against deionized, distilled water and tested for their growth factor activity. A representative column profile is shown in this figure. EC number (○) is the average of triplicate wells; 3T3 cell [^{3}H]thymidine incorporation (●) is the average of two wells.

the immobilized heparin (data not shown). Application of the RDGF, partially purified by heparin-Sepharose chromatography, to an HPLC TSK 2000 size exclusion column yielded the profile shown in Fig. 3. One peak of growth factor activity was obtained and was eluted with a molecular weight of ~18,000.

The combination of heparin-affinity chromatography with either IEF or size exclusion chromatography resulted in the isolation of a highly purified growth factor activity. Table I illustrates the results of analysis of RDGF by heparin-Sepharose and size exclusion. This scheme increased the specific activity of the growth factor 15,000-fold from 133 U/mg to 2

$\times 10^6$ U/mg. At this level of purity, RDGF stimulated EC proliferation at concentrations of 1–10 ng/ml. SDS PAGE analysis with silver stain visualization was performed on RDGF purified by heparin-Sepharose and TSK 2000 size exclusion chromatography. About 10 bands were evident, including a predominant band that migrated with a molecular weight of 17,800. This band is a likely candidate for RDGF based on the elution of growth factor activity from an HPLC TSK 2000 size exclusion column.

Comparison of RDGF and aHDGF

The EC growth factor from hypothalamus has been reported to have an isoelectric point of approximately 5.0 (19). We have confirmed this finding by IEF (data not shown). Separation of both RDGF and aHDGF by IEF both gave a single peak of growth factor activity with a pI of 5.0. The peaks of these activities obtained by IEF were applied to separate columns of heparin-Sepharose. Fig. 4 shows that both RDGF and the aHDGF activity bound to heparin and eluted at the same NaCl concentration (0.9–1.1 M). Both growth factors stimulated DNA synthesis by 3T3 cells (Fig. 4, top) as well as the proliferation of capillary endothelium (Fig. 4, bottom). When RDGF and the aHDGF prepared by heparin-Sepharose chromatography were analyzed by size exclusion chromatography, the growth factor activities eluted in exactly the same fractions; this result indicates that they are of similar molecular size, approximately 18,000 (Fig. 5).

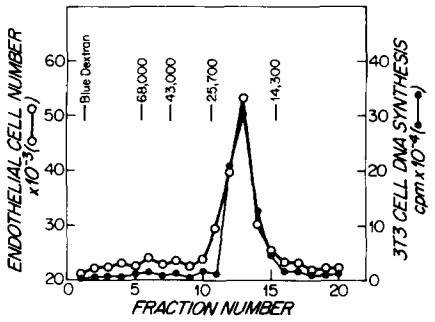

FIGURE 3 Size exclusion chromatography of RDGF obtained from heparin-Sepharose. The peak of growth factor activity that was eluted from heparin-Sepharose between 0.9 and 1.1 M NaCl was dialyzed against deionized, distilled water, lyophilized, and resuspended in 100 μl of 0.6 M NaCl, 0.02 M Tris-HCl, pH 7.0. The growth factor was then fractionated on a TSK 2000 size exclusion column equilibrated in the same buffer at room temperature with a flow rate of 0.5 ml/min. Fractions (0.8 ml) were collected, dialyzed against deionized, distilled water, and tested for growth factor activity. A representative column profile is shown in this figure. EC number (○) is the average of triplicate wells; 3T3 cell [^{3}H]thymidine incorporation (●) is the average of two wells. The elution positions of molecular weight standards are indicated.

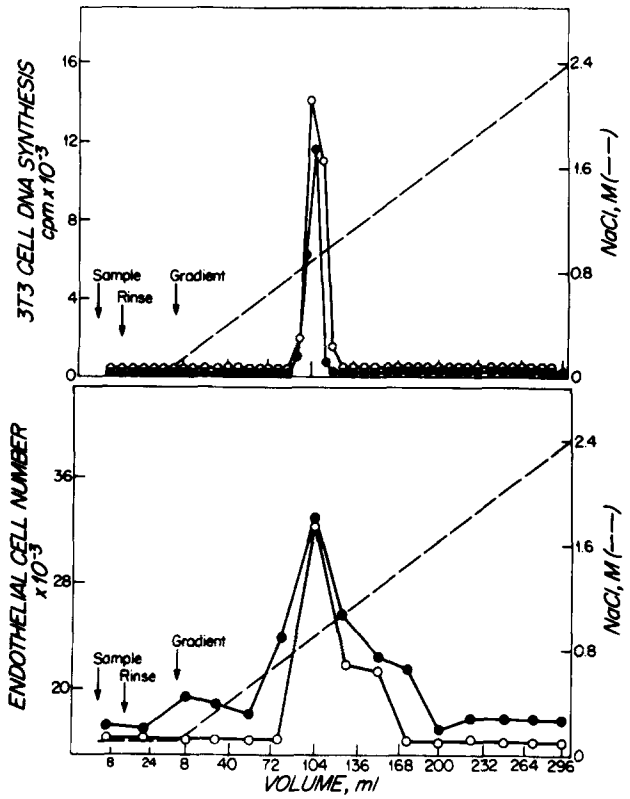

FIGURE 4 Comparison of RDGF and aHDGF by heparin-sepharose affinity chromatography. RDGF (30 ml) and aHDGF (16 ml) activity that focused at pH 5.0 were dialyzed against 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, and applied to separate heparin-Sepharose columns (1 × 7 cm). After rinsing (50 ml for RDGF, 40 ml for aHDGF) with equilibration buffer, a gradient of 0.1 to 3.0 M NaCl (200 ml for RDGF, 320 ml for aHDGF) was applied and the protein was eluted from the column at 40 ml/h. The fractions were dialyzed against deionized, distilled water, lyophilized, and tested for growth factor activity. Representative column profiles are shown in this figure. The top panel compares RDGF (○) and aHDGF (●) stimulation of [^{3}H]thymidine incorporation by 3T3 cells; thymidine incorporation is the average of duplicate wells. The bottom panel compares RDGF (○) and aHDGF (●) stimulation of EC proliferation; cell number is the average of triplicate wells.

DISCUSSION

We have previously demonstrated that an EC mitogen could be extracted from the retina by incubation in a balanced salt solution (10). Subsequent studies demonstrated that the activity was protease-sensitive and bound to DEAE cellulose (5) but not to CM cellulose (D'Amore, P. A., unpublished results). Together, these results indicate that the active molecule is an anionic polypeptide. Earlier estimates of molecular weight based on behavior during dialysis and ultrafiltration studies

TABLE I
Purification of RDGF

	Protein*	Activity	Specific activity	Recovery	X-fold purification
	mg	*U*†	*U/mg*	%	
Crude extract (10 ml)	60	8,000	133	100	1
Heparin-Sepharose	0.009	4,400	490,000	55	3,600
HPLC TSK 2000	0.0009	1,800	2×10^6	27.5	15,000

* Protein concentration of the crude extract and the heparin-Sepharose-purified activity was determined by Lowry. Comparative density on silver stained SDS polyacrylamide gels was used for protein determination of the HPLC TSK-purified material.
† A unit is defined as the amount of growth factor required to give half-maximal [^{3}H]thymidine incorporation by 3T3 cells.

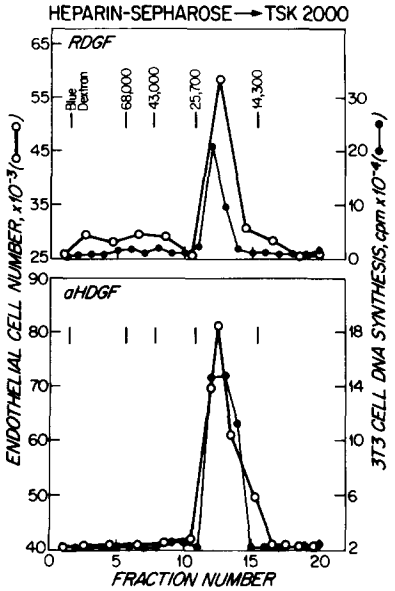

FIGURE 5 Comparison of RDGF and aHDGF by HPLC size exclusion chromatography. The peaks of RDGF and aHDGF activity which were eluted from heparin-Sepharose by 0.9–1.1 M NaCl (Fig. 4) were dialyzed against deionized, distilled water, lyophilized, and then resuspended each in 100 μl of 0.6 M NaCl, 0.02 M Tris-HCl, pH 7.0. The growth factors activities were then fractionated on a TSK 2000 size exclusion column (Varian, 8 mm I.D. × 50 cm) equilibrated in the same buffer at room temperature with a flow rate of 0.5 ml/min. Fractions (0.8 ml) were collected, dialyzed as above, and tested for their growth factor activity. Representative column profiles are shown in this figure. The top panel illustrates the effect of RDGF on capillary endothelial cell proliferation (O) and [^{3}H]thymidine incorporation by 3T3 cells (●). The bottom panel is a parallel experiment conducted with aHDGF. EC number is an average of triplicate wells; 3T3 cell [^{3}H]thymidine incorporation is the average of two wells. The elution positions of the molecular weight standards are indicated.

indicated a molecular weight of >50,000. Whether this larger molecular weight form is due to nonspecific associations or the existence of a specific carrier molecule is unknown. However, the tendency of the activity to aggregate made column chromatography in low salt buffers difficult. Only in presence of high concentrations of chaotropic agents such as guanidine was significant resolution of the activity accomplished. With HPLC size exclusion chromatography in 6 M guanidine, the growth factor activity eluted from the column with a molecular weight between 16,000 and 22,000 (6, 22) yielding only a ninefold purification with 50% recovery (6). In contrast, the high affinity of RDGF for heparin has allowed greater than 3,500-fold purification with 55% recovery (Table I). Heparin-Sepharose chromatography in conjunction with size exclusion chromatography or IEF permits a rapid 15,000-fold purification of this potent endothelial mitogen.

The data presented here on the analysis of RDGF by IEF confirms that the molecule is anionic. In light of this, the binding of the anionic growth factor to another anionic molecule, heparin, was unexpected. However, a precedent for an interaction of this nature occurs in the binding of platelet factor four (pI 7.6) to heparin. Handin and Cohen (13) comment that the heparin-platelet factor four interaction involves more than "the neutralization of charge by the combination of a positively charged protein with a highly negative aminoglycan" (13). Both the EC surface (3) and its basal lamina (14) contain heparin-like molecules. The binding of RDGF to these molecules would represent a possible mechanism for the concentration of growth factors by cells in vivo. The potential significance of the growth factor–heparin interaction is emphasized by the recent finding of Thornton et al. (24) who reported that the addition of heparin to the culture medium potentiates the stimulatory effect of an EC mitogen.

The finding that the anionic molecule RDGF bound to heparin prompted us to investigate whether another anionic endothelial mitogen would similarly adhere to heparin. It has been demonstrated that the hypothalamus contains an anionic (pI of 5) factor that stimulates Swiss 3T3 cells as well as human umbilical vein EC (18). This factor is reported to exist in both a high (75,000) and low (15,000) molecular weight form (19). In our studies, comparison of aHDGF and RDGF revealed three major biochemical similarities: (*a*) isoelectric points of 5.0, (*b*) co-elution from heparin-Sepharose at salt concentrations of 0.9–1.1 M NaCl, and (*c*) co-migration on size exclusion columns in the molecular weight range of 18,000. In addition, RDGF and aHDGF exhibit functional similarities. Both growth factors are mitogenic for capillary as well as human umbilical vein endothelium (6, 18). Further, neither RDGF (10) nor aHDGF (Klagsbrun, M., unpublished results) stimulates the proliferation of vascular smooth muscle cells, indicating a target cell specificity. On the basis of these characteristics, we suggest that the two factors isolated from two different tissues are very similar, if not identical molecules. The pituitary (9) and brain (23) appear also to contain an anionic growth factor, acidic fibroblast growth factor, which is similar to RDGF and aHDGF. Several characteristics of this growth factor, including its isoelectric point (pI of 5), its molecular weight (~16,000), and its ability to stimulate EC and 3T3 cell proliferation indicate a high degree of similarity if not identity with RDGF and aHDGF.

RDGF has been demonstrated to stimulate the proliferation of new blood vessels in two in vivo assay systems (5, 10). We had postulated that RDGF might function in the retina to induce the new capillary formation that characterizes pathologies such as diabetic retinopathy. As of yet, the biosynthetic site of RDGF is unknown. The existence of similar or identical growth factors in four tissues, the retina, the hypothalamus, the brain, and pituitary, suggests an exciting possibility: that RDGF may be synthesized in the hypothalamus or pituitary and then transported to the retina. Several lines of evidence support this theory. First, it is well documented that diabetic dwarfs do not develop proliferative diabetic retinopathy (20). This suggests that normal pituitary-hypothalamus function is a prerequisite for the development of retinal vessel proliferation. Second, hypophysectomy has been reported to reduce the blood vessel growth associated with diabetic retinopathy (2), again indicating that an upset of normal hypothalamo-hypophyseal interaction removes the stimulus for new blood vessel formation. Third, there is an intimate vascular association at the level of the optic chiasm that connects the optic nerves, the retina, and the hypothalamohypophyseal system. This vascular connection could provide the means for the transport of the growth factor/hormone from its site of synthesis to the retina where it could be stored, further processed, destroyed, or transported. Alternatively, since the retina, hypothalamus, pituitary (9), and brain (Klagsbrun, M., unpublished results) (15, 23) all appear to contain similar growth factors, is that these activities may belong to a class of growth factors that are common to all tissues of neural origin.

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