THE USE OF COLLAGENASE FROM THE HEPATOPANCREAS OF THE KAMCHATKAN CRAB *Paralithodes camtschatica* TO ISOLATE AND CULTURE ENDOTHELIAL CELLS FROM THE HUMAN UMBILICAL VEIN

N. V. Goncharov, I. Yu. Sakharov,
S. M. Danilov, and O. G. Sakandelidze

UDC 612.13.014.2-085.23:57.086.82/.83.
577.152.344

KEY WORDS: collagenase; endothelium; blood vessels; cell culture.

Our ideas on the structure and function of endothelial cells lining the luminal surface of blood vessels have widened considerably [4]. This is due primarily to progress in the in vitro culture of these extraordinarily "capricious" cells, which was largely due to the use of collagenase instead of trypsin as the proteolytic enzyme for removing these cells from the surface of large blood vessles [8]. Collagenase can also be used to remove cells from the substrate during subculture. Collagenase has been found to cause much less damage to the endothelial cell membrane than trypsin, which is traditionally used for these purposes [5]. The main source of collagenase at present is a pathogenic strain of *Clostridium histolyticum*. The production of microbiological collagenase (MC) is beset by considerable difficulties because of the pathogenicity of the strain, and for that reason commercial preparations of MC are expensive. The crab hepatopancreas can be used as an alternative source of collagenase [2].

The aim of this investigation was to study the possibility of using collagenase from the hepatopancreas, a waste product of commercial fishing of the Kamchatkan crab *Paralithodes camtschatica* and large human blood vessels and to subculture these cells in vitro.

EXPERIMENTAL METHOD

The hepatopancreas of *Paralithodes camtschatica* was used. Collagenases were isolated by the method in [2] in the Laboratory of Biotechnology, Pacific Institute of Bioorganic Chemistry, Far-Eastern Scientific Center, Academy of Sciences of the USSR. The specific activity of collagenase from the crab hepatopancreas (CHC) relative to acid-soluble collagen is 93 U/mg of the preparation. The preparation contained 17% of protein. Activity relative to elastin and the ethyl esters of N-acetyl-L-tyrosine and N-benzoyl-L-arginine has not been found.

Isolation and culture of endothelial cells from the human umbilical vein were carried out by the method in [3] with some modification. The umbilical vein was washed under sterile conditions with Hanks' medium 199 with salt (Institute of Poliomyelitis and Virus Encephalitis, Academy of Medical Sciences of the USSR), with the addition of antibiotics. The umbilicus was then cut into two halves and the vein of each half was immersed in 0.1% solution of CHC or MC (type 11, 131 U/mg preparation, from Worthington, USA), preheated to 37°C and incubated for 15 min at room temperature. The suspension of detached cells was washed from the vein into a centrifuge tube and centrifuged for 5 min at 500g. The residue was resuspended in complete growth medium and transferred into culture flasks (Nunc, Denmark), coated before hand with a 1% solution of gelatin (Sigma, USA). The complete growth medium consisted of medium 199 with Earle's salts (Gibco, USA), with 20% of pooled human serum heated for 40 min to 56°C, 4 mM glutamine (Gibco), 15 mM HEPES buffer (Flow Laboratories, England), 100 U/ml of penicillin, 100 μg/ml streptomycin (Gibco), 100 μg/ml heparin (Sigma), and 200 μg/ml of endothelial cell growth factor, isolated from human brain [1] by the method

Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR. Research Laboratory of Biologically Active Substances of Hydrobionts, Ministry of Health of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Smirnov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 104, No. 9, pp. 376-378, September, 1987. Original article submitted June 10, 1986.

 0007-4888/87/0009-1324$12.50

TABLE 1. Isolation of Endothelial Cells from the Human Umbilical Vein with the Aid of Different Collagenase Preparations

Collagenase preparation	Exp. No.	Total number of cells* ($\times 10^3$)	No. of living cells* ($\times 10^3$)	Number of living cells, (%)
MC (0,1%)	1	570	390	69
	2	2070	1870	90
	3	1300	1050	82
	4	790	247	31
	5	950	650	68
	6	1030	640	63
CHC (0.1%)	1	640	295	52
	2	2170	1640	53
	3	1500	470	32
	4	1190	190	16
	5	730	290	40
	6	580	90	16

Legend. Here and in Table 2, asterisk denotes mean of two independent determinations.

TABLE 2. Subculture of Endothelial Cells from the Human Umbilical Vein with the Aid of Different Proteolytic Enzymes

Enzyme	No. of detached cells* ($\times 10^3$)	Percentage of living cells	Time of removal of cells from substrate
0.125% trypsin + 0.02% EDTA	547	95	40 sec
0.1% CHC + 0.02% EDTA	520	92	90 sec
0.1% MC + 0.02% EDTA	250	—	15 min

in [6]. The viability of the cells was determined by staining with trypan blue. The number of cells was counted in a hemocytometer.

EXPERIMENTAL RESULTS

In the experiments of series I we tested the possibility of using CHC to isolate endothelial cells from large blood vessels with particular reference to the human umbilical vein. The total number of detached cells was virtually the same in each case and varied from $0.5 \cdot 10^6$ to $2 \cdot 10^6$ depending on the length of the umbilicus (Table 1). The mean percentage of viable endothelial cells when MC was used was 67, compared with 35 when CHC was used. Reducing the CHC concentration to 0.03% reduced the number of detached cells sharply (by 60%). After isolation from the umbilical vein the suspension of endothelial cells in complete growth medium was transferred into culture flasks with an area of 21 cm^2, at the rate of 100,000-200,000 cells per flask. The use of endothelial cell growth factor and a gelatin substrate enabled these cells to be grown from culture from low density to a monolayer in a short period of time, and throughout many subcultures [1]; the doubling time of the population was 20-25 h. Differences in the number of viable cells in the course of isolation were thus virtually eliminated at the first passage. During subsequent culture endothelial cells isolated with the aid of MC and CHC did not differ in their rate of growth, cell morphology in the monolayer, and the presence of the most important endothelial markers (factor VIII, angiotensin-converting enzyme activity).

In the experiments of series II the possibility of using CHC to detect the endothelial cells substrate during subculture was studied. In control experiments, MC and trypsin were used for this same purpose. All the enzymes were dissolved in versine in the ratio of 1:5000 (Institute of Poliomyelitis and Virus Encephalitis, Academy of Medical Sciences of the USSR). The results are given in Table 2.

The low percentage of detached endothelial cells obtained by the use of MC can evidently be explained by the fact that the molecule of this enzyme contains a Ca^{++} ion [7]. In the presence of EDTA the ion of this metal is extracted from the active center, with the formation of an apoenzyme, which leads to inactivation of MC. In contrast to MC, CHC activity was unchanged in the presence of 2.4 mM EDTA or 2.5 mM o-phenanthroline. Ca^{++}, Cd^{++}, Mn^{++}, Mg^{++}, and Hg^{++} ions in a concentration of 25 mM likewise have no appreciable effect on CHC activity. Taken together, these data explain the high efficacy of CHC for the removal of endothelial cells from the substrate.

Trials of the CHC preparation showed that it is virtually not inferior in collagenolytic activity to collagenase obtained from the firm "Worthington" and is suitable for work with cultures of endothelial cells — one of the most delicate and "capricious" test objects. This preparation can be used on a large scale, because the method of its preparation is exceedingly simple and inexpensive, and what is no less important, it does away with the need to work with a pathogenic raw material.

LITERATURE CITED

1. S. M. Danilov, E. Y. Allikmets, and A. V. Martynov, J. Cell. Biol., 99, 274A (1984).
2. A. Z. Eisen, K. O. Henderson, J. J. Jeffrey, and R. A. Bradshaw, Biochemistry, 12, 1814 (1973).
3. E. A. Jaffe, R. L. Nachman, C. C. Becker, and C. R. Minick, J. Clin. Invest., 52, 2745 (1973).
4. E. A. Jaffe, Biology of Endothelial Cells, ed. by E. A. Jaffe, Boston (1984), p. 1.
5. C. J. Kirkpatrick, I. Melzner, and T. Goller, Biochim. Biophys. Acta, 846, 120 (1985).
6. T. Maciag, Proc. Nat. Acad. Sci. USA, 76, 5674 (1976).
7. S. Seiffer and E. Harper, Methods Enzymol., 19, 613 (1970).
8. D. G. S. Thilo-Korner, D. Heinrich, and H. Temme, The Endothelial Cell — A Pluripotent Control Cell of the Vessel Wall, ed. by D. G. S. Thiol-Korner and R. I. Freshney, Besel (1982), p. 158.

A METHOD OF STUDYING RESPONSES OF SINGLE VESSELS IN SITU

V. P. Nikol'skii and A. N. Rogoza

UDC 616.13./14-009.1-07:/616.13/.14-073.173:616.151.4

KEY WORDS: blood vessel; hydraulic resistance.

A very important parameter of the functional state of blood vessels and of changes in that state is hydraulic resistance. This can be calculated by known equations [2], given the geometric dimensions of the vessel, the viscosity of the blood, and the character of its flow. To record changes in the diameter of blood vessels various methods have been developed, based on the use of contact diameter transducers [4], miniature ultrasonic transducers fixed to the vessel wall [9], television systems [10], and x-ray or ultrasonic angiography [8]. Some of these methods are applicable only to superficial vessels or they require quite traumatic dissection, whereas others cannot guarantee sufficient accuracy of measurement of the internal diameter of blood vessels under 1 mm in caliber. An alternative method of determining resistance is based on measurement of the pressure drop on the vessel and the blood flow along it [7].

The method we have developed consists essentially as follows. Blood flowing along the vessel is passed through a standard hydraulic resistance (standard) and returned to the

Laboratory of Biomechanics and Regulation of the Circulation, Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow.

(Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Smirnov.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 104, No. 9, pp. 378-381, September, 1987. Original article submitted December 5, 1986.

0007-4888/87/0009-1326$12.50