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Hyaluronidases and their inhibitors in the serum of colorectal carcinoma patients

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ABSTRACT

Colorectal cancer is the third most commonly diagnosed type of cancer. Hyaluronan is involved in this malignancy. Moreover, hyaluronidases – its degrading enzymes – display controversial roles regarding their involvement in tumor development. HYAL-1 is the major tumor derived hyaluronidase. The aim of the study was the determination and evaluation of hyaluronidase levels in serum of colorectal cancer patients, before and after surgery, with a view to assessing its potential role as a tumor marker for recurrence. By zymography and Western blotting, it was confirmed that HYAL-1 was the only hyaluronidase present in samples. Quantification of its activity indicated a statistically significant decrease in samples seven days postoperatively, compared with the respective ones before surgery. HYAL-1 levels before surgery were significantly reduced in comparison with healthy samples and samples one year postoperatively. Hyaluronidase inhibitor activity was demonstrated under mild alkaline conditions via reverse zymography. A statistically significant increase was observed in samples seven days postoperatively, when compared with samples before surgery. HYAL-1 levels in sera of colorectal cancer patients were lower than those of healthy population, possibly because of the local accumulation of the enzyme in tumor microenvironment. A gradual elevation up to one year postoperatively to reach healthy levels might indicate a role of HYAL-1 levels in cancer.

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1. Introduction

Colorectal cancer (CRC), being responsible for 655,000 deaths worldwide per year, is the third most commonly diagnosed type of cancer [1]. Most colorectal cancers occur due to lifestyle and increasing age with only a minority of cases associated with underlying genetic disorders. Familial adenomatous polyposis and hereditary non polyposis colorectal cancer illustrate such disorders.

As far as molecular origin of CRC is concerned, mutations in tumor suppressor genes and oncogenes have been implicated in tumor growth and progression [2]. Such genes have been utilized as molecular markers in diagnosis and prognosis. Genetic testing is deemed as an absolutely indispensable procedure for detecting predisposition for CRC in high risk candidates, such as patients with inherited polyposis and non polyposis syndromes. However, the most effective screening method remains colonoscopy, which simultaneously provides the opportunity of removing suspected lesions. Carcinoembryonic antigen (CEA) is applied as a blood tumor marker to identify metastasis or recurrence after surgical resection [2].

Hyaluronan (HA) – a glycosaminoglycan of extracellular matrix (ECM) composed of N-acetylglucosamine and glucuronic acid repeats – is normally involved in embryogenesis, morphogenesis, wound healing, etc [3]. In such occasions its levels rise. Nevertheless, its levels elevate also in pathological conditions including several types of cancer and a high level of pericellular HA in tumors is a strong indicator of unfavorable patients' outcome. In CRC, more than 80% of the tumor cells are HA positive [4].

Hyaluronidases (Hyals) are a class of enzymes which predominantly degrade HA. In the human genome there are six known genes coding for HYAL-like sequences [5]. Among them, lysosomal HYAL-1 is expressed in several somatic tissues and concurrently is the serum HYAL [6]. Although the exact tissue source of this circulating enzyme remains elusive, it may be considered that it stems from white blood cells, mainly monocytes [7]. Three other active HYALs exist, HYAL-2, HYAL-3 and PH-20 [5], none of which is present in serum. HYALs display a controversial role regarding their

Abbreviations: HA, hyaluronan/hyaluronic acid; HYAL, hyaluronidase; CRC, colorectal cancer; CEA, carcinoembryonic antigen; BSA, bovine serum albumin.

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Table 1
Clinocopathological characteristics of the patients included in the study.

No	Age	Sex	Anatomic site	TNM stage	A/C stage	Grade
1	59	M	Rectum	T2N1	C1	Median
2	80	F	Ascending	T3N1	C2	Poor
3	71	M	Descending	T3N1	C2	Median
4	82	F	Descending	T3N0	B2	Median
5	79	M	Rectum	T3N0	B2	Well
6	72	F	Transverse	T1N0	A	Well
7	70	F	Ascending	T3N1	C1	Poor
8	77	M	Rectum	T3N0	B2	Poor
9	58	M	Rectum	T2N0	B1	Median
10	60	F	Ascending	T3N0	B2	Poor
11	77	M	Sigmoid	T2N0	B2	Median
12	79	M	Cecum	T3N0	B2	Median
13	50	M	Sigmoid	T3N1	C2	Median
14	66	M	Sigmoid	T3N0	B2	Median
15	81	M	Sigmoid	T3N0	B2	Median

involvement in tumor development. However, it has been shown that this is a concentration dependent behavior [8] at least for the majority of occasions, since different cancers utilize distinct strategies in order to be promoted. HYAL-1 is the major tumor derived HYAL and is expressed by a variety of tumor cells.

The presence of HYAL-1 in genitourinary cancers has been proposed for diagnosis [9]. Its increased levels in serum and especially in urine correlate with the cancer state, and thus it can be used as a biochemical marker [10]. In addition, it has been proposed as potential prognostic indicator for progression to muscle invasion and recurrence in bladder cancer [11]. Elevated levels of HYALs have also been observed in other types of cancer both in vivo and in vitro [12–18]. Nonetheless, apart from ovarian cancer there is no clear evidence concerning their levels in serum. The purpose of conducting this specific study was to determine and evaluate HYAL levels in serum samples of CRC patients postoperatively, with a view to assessing its potential as a tumor marker in CRC.

2. Materials and methods

2.1. Biological samples

Serum samples were obtained from six healthy volunteers, aged between 45 years and 78 years, five patients with adenomas, aged between 59 years and 82 years, and fifteen patients with CRC, aged between 56 years and 86 years, having undergone surgical treatment, performed at Surgical Clinic of the General University Hospital of Patras, Greece. Sample collection for each patient was carried out one day prior to their operation and at intervals of one week, one month, three months, six months and one year postoperatively. Samples were stored at −20 °C and −80 °C for short-term and long-term use, respectively. Clinical information for each patient is summarized in Table 1. The study design was approved by the Ethical Committee of the University Hospital of Patras, Greece, and informed consent was obtained from all patients entering the study.

2.2. Reagents

Hyaluronic acid sodium salt from *Streptococcus Zooepidemicus* and N-acetyl-D-glucosamine were purchased from Sigma Chemical Co (St Louis, MO, USA). Hyaluronidase from bovine testes (type I-S), Pronase from *Streptomyces griseus* and goat anti-mouse peroxidase conjugated antibody were also from Sigma Chemical Co (St Louis, MO, USA). Alcian blue and Coomassie brilliant blue R-250 were obtained from Serva Biochemicals (Darmstadt, Germany). Mouse polyclonal antibodies against HYAL-1, HYAL-2, HYAL-3 and PH-20/SPAM1 were purchased from Abnova (Taiwan). PVDF Immobilon P was obtained from Millipore (Dorset, UK). Enhanced chemiluminescence (ECL) Western blotting substrates were from Pierce Biotechnology (Rockford, IL, USA). All other chemicals were of analytical grade.

2.3. Detection of hyaluronidase activity and hyaluronidase inhibitors

2.3.1. Zymography

Substrate gel electrophoresis for verifying HYAL activity was applied essentially as previously described [19]. In particular, human serum samples were applied to 10% gels containing 0.1 mg/ml HA, at initial sample dilutions ranging from 0.5% to 20% (v/v) with ddH_2O. Experiments were also carried out under reducing conditions in the presence of β-mercaptoethanol. Following the end of electrophoresis the gels were incubated for 45 min in a 3% Triton X-100 solution for renaturation of enzymes and subsequently in optimal Hyal buffers at pH 3.7, 5.2 and 7.4 at 37 °C for 16 h. Then, the gels were processed with pronase solution at 37 °C for 4 h and eventually stained in Alcian blue and Coomassie solutions according to the protocol. Semi-quantification of enzymatic activity was attained by utilizing Scion Image software.

Regarding detection for HYAL inhibitors activity inverse HA substrate gel electrophoresis was performed according to the procedure described by Mio et al. [19]. Five μl of serum were used after mixing with an equal volume of sample buffer (2×). Various buffers were tested for optimal results.

2.3.2. Quantification of reducing N-acetyl-D-glucosamine

Measurement of HYAL activity was determined using Reisig's modified colorimetric method for the estimation of N-acetyl-D-glucosamine (GlcNAc) being released following HA digestion [12,20]. Four μl of serum were diluted in appropriate buffer to obtain a final solution of 200 μl comprising 40 μg HA. HYAL activity was measured according to a GlcNAc standard curve (2.5–10 μg, equal to 11.3–45.2 μmol) and was expressed as μU. As 1 U of HYAL activity was defined: (produced μmol GlcNAc)/(min of reaction) under the experimental conditions.

Concerning the quantification of Hyal inhibitors activity a variation of the aforementioned protocol was utilized without altering the fundamentals of the method. Particularly, commercially available bovine Hyal was added in the initial reaction solution at a concentration of 0.2 U/μl. Suitable buffer solution providing mild alkaline conditions (50 mM HEPES, 150 mM NaCl, 0.2 mg/ml BSA, 1 mM $MgCl_2 \cdot 6H_2O$ pH 7.4) replaced the respective acidic one. Hence, the only contributory factor/enzyme in HA degradation would be the exogenously added Hyal and not the endogenous one which is utterly deactivated at this pH. In essence potential decrease in total Hyal activity would be translated to proportional presence of Hyal inhibitor action in the biological sample the volume of which was 5 μl of undiluted serum. Incubation time for reaction lasted 3 h instead of 24 h. All the other steps of the procedure remained the same.

2.4. Western blotting

Following their separation via 10% SDS–PAGE electrophoresis under reducing conditions imposed by β-mercaptoethanol, serum constituents were electrophoretically transferred to PVDF membranes at constant current for 2 h (200 mA the first hour and 250 mA the second one). Then a 3% solution of bovine serum albumin (BSA) in 140 mM sodium chloride–10 mM sodium phosphate pH 7.4 containing 0.1% Tween-20 (PBST) was used as the blocking agent and the membranes were probed by the primary antibody solution (against HYAL-1, HYAL-2, HYAL-3 and PH-20/SPAM1) overnight at 4 °C. Primary antibody was applied at a concentration 1/2000

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