Activity and Subunit Composition of Proteasomes in Head and Cervical Squamous Cell Carcinomas

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The development of malignant neoplasm of the head and neck is related to the state of intracellular proteasome system. Elevation of total activity of proteasomes and specific activity of 20S proteasomal pool are accompanied by changes in proteasomal composition. Tumor size correlated with the content of regulatory proteasomal complex PA28. In the presence of regional metastases, 26S proteasome activity decreases and the content of proteasome immune subunit LMP7 in the tumor increases.

Key Words: *head and neck squamous cell carcinomas; proteasomes; proteasome immune subunits; proteasomal regulators*

In studies of the mechanisms of malignant growth, an important role is given to proteinases, which are not only the protein degrading enzymes, but also regulators of vital cell processes. Intracellular selective hydrolysis of the proteins involved in numerous processes controlling the cell life are performed by the proteasomes demonstrating trypsin-like, chymotrypsin-like, and caspase activities. To study these activities, the researches focused on 20S and 26S proteasomes [7,11]. As a rule, proteolysis is carried out in 26S proteasome, although 20S pool also possesses catalytic activity and hydrolyses peptides and proteins with damaged tertiary structure [3,9]. The 26S proteasome includes 20S proteasomes and one or two regulatory subcomplexes 19S (PA700). If only one subcomplex 19S is bound to 20S proteasome, the latter can bind the second regulatory complex such as PA28 [11].

Proteasomes exist in the constitutive and immune forms. The immune proteasomes contain catalytic subunits LMP7 (β5i), LMP2 (β1i), and MECL1 (β2i) instead of catalytic subunits X (β5), Y (β1), and Z (β2) of constitutive proteasomes. Replacement of the constitutive subunits with immune ones modifies enzyme activity [4]. The constitutive 26S proteasomes are involved in the regulation of cell processes, while the immune 26S proteasomes are needed to arrange the immune response and to produce the major histocompatibility class I complexes [3,11]. In addition, the immune proteasomes are involved in the regulation of cell differentiation [12], proliferation [6], and antioxidation activity [10].

Dysfunction of the proteasome system is characteristic of many pathologies and plays a significant role in the development of tumor diseases. The experiments revealed reduced content of proteasome immune pools in ascitic carcinoma Krebs-II cells compared to normal tissues [2]. The content of proteasomes and their activity in human malignant tumors received little attention. Specifically, there isare no data on these proteolitic copmplexes in head and neck squamous cell carcinomas (HNSCC).

Our aim was to study proteasomal activity and subunit composition of the proteasomes in HNSCC tissue and their correlation with clinical and morphological indices of the disease.

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MATERIALS AND METHODS

The study enlisted the patients aging 57.70±2.19 (n=25) with head and neck tumors ($T_{2-4}N_{0-3}M_0$). We examined specimens of the tumor and visually unchanged tissue (taken at a distance >1 cm from the tumor boundary) obtained during surgery. All tumors demonstrated the histological structure of HNSCC with various degree of differentiation.

The clarified homogenates were obtained as follows. Frozen tissue (100 mg) was homogenized in liquid nitrogen and then resuspended in 300 µl Tris-HCl buffer (50 mM, pH 7.5) containing (in mM): 2.0 ATP, 5.0 $MgCl_2$, 1.0 dithiothreitol, 1.0 EDTA, and 100 NaCl. The homogenate was centrifuged at 10,000g and 4°C for 60 min.

All procedures were performed at 4°C. The proteins of clarified homogenates were fractionated with ammonium sulfate in two stages. The fraction enriched with 26S proteasomes was obtained by adding ammonium sulfate to 40% saturation and 20S fraction was obtained by adding this agent to 70% saturation [1].

Total activity of proteasomal pool containing both 26S and 20S fractions was determined in homogenates of the tumor and normal tissue by hydrolysis of fluorogenic oligopeptide Suc-LLVY-AMC, which is degraded by the chymotrypsin-like proteasomal centers [5]. The reaction mixture used to determine activity of total proteasomal pool and that of 26S proteasomal pool contained (in mM): 20.0 Tris-HCl at pH 7.5, 1.0 dithiothreitol, 0.03 Suc-LLVY-AMC, 5.0 $MgCl_2$, and 1.0 ATP. The reaction mixture used to determine activity of 20S proteasomal pool had the same composition except $MgCl_2$ and ATP. The reaction was carried out for 20 min at 37°C. Activity of the resulting product was measured on a Hitachi-850 fluorimeter at excitation wavelength 380 nm and emission wavelength 440 nm. The amount of enzyme hydrolyzing 1 nM Suc-LLVY-AMC over 1 min was taken as 1 U of proteasomal activity. Specific proteasomal activity was determined in units of activity per 1 mg protein. Protein content was measured by the method of Lowry.

Electrophoresis was carried out according to the method of Laemmly in 13% PAAG. The probes were tested in a buffer containing 0.0625 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue.

After electrophoresis, the polypeptides were transferred onto Hybond-ELC nitrocellulose membrane (Amersham). The membrane was incubated for 2 h at 20°C in TNT buffer containing 10 mmol Tris-HCl at pH 7.5, 150 mM NaCl, and 0.1% Tween-20. Then the membrane was incubated in the same buffer supplemented with 5% skim milk and the monoclonal antibodies raised against α1α2α3α5α6α7, LMP7, LMP2, or PA28β proteasomes diluted to 1:2500. Thereafter the membrane was several times washed in TNT buffer and incubated for 1 h in TNT buffer with 5% skim milk and antibodies against mouse IgG conjugated with peroxidase and diluted to 1:10,000. After washing, the membrane was routinely processed in the chemoluminescent protein detection system (Amersham). The band density was determined using Image J software. The results were presented in percentage of subunit content in the unchanged tissue taken for 100%.

The data were processed statistically using Mann–Whitney non parametric U test and Statistics 6.0 software.

RESULTS

In HNSCC patients, total activity of proteasomes and activity of 20S proteasomes in the tumor surpassed the corresponding values in the adjacent unchanged tissue (Fig. 1). Probably, the increase in total proteasomal activity in tumor results from enhancement of 20S proteasome activity. It is known that 20S proteasome can degrade small peptides and damaged proteins, which is probably important for the functioning of tumor cells [8].

Figure 2 shows subunit composition of proteasomes in HNSCC obtained by Western blot immunoassay. In tumor tissue, the content of α1α2α3α5α6α7 subunits decreased to 69.1±12.3% compared to the corresponding level in unchanged tissue (p<0.05). At the same time, the content of immune subunits LMP7 and LMP2, as well as subunit PA28β in the tumor was higher than in unchanged tissue: 220.4±24.5, 154.4±14.6, and 147.4±10.1% of the corresponding values

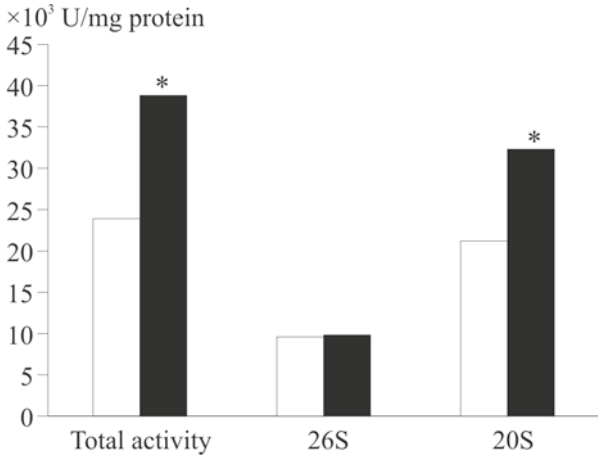

Fig. 1. Comparison of total and fractional proteasomal activity between tumor (closed bars) and unchanged (open bars) tissue during HNSCC. Here and in Fig. 2: *p<0.01 compared to unchanged tissue.

TABLE 1. Correlation between Activity and Subunit Composition of Proteasomes and Clinical and Morphological Indices of HNSCC (M±m)

Clinical morphological indices		N	Activity of proteasomes in tumor tissue, $\times 10^3$ U/mg protein			Content of proteasomes in tumor tissue, % of content in unchanged tissue			
			total	26S	20S	α1α2α3 α5α6α7	LMP7	LMP2	PA28β
Tumor size	T_2	12	35.7±4.3	10.4±2.2	31.1±4.9	52.8±8.4	236.4±43.3	145.4±24.5	129.4±16.8
	T_{3-4}	14	41.6±7.8	9.3±2.1	33.4±9.3	80.9±20.0	208.7±29.7	161.1±18.6	160.6±11.5*
Regional tumor dissemination	$T_{2-4}N_0$	15	40.3±7.4	12.4±2.2	39.8±9.8	61.1±10.3	163.9±21.1	142.7±11.8	148.5±14.9
	$T_{2-4}N_{1-3}$	11	36.9±4.5	7.2±1.7+	24.7±4.0	76.4±21.9	271.3±36.4+	164.9±25.6	146.4±14.5
Differentiation degree	poorly differentiated	5	34.8±6.7	9.8±3.4	24.0±7.4	62.6±37.0	284.9±46.5	147.15±38.6	162.2±23.5
	moderately differentiated	14	42.7±8.0	8.0±1.6	37.0±10.0	78.9±16.8	182.2±32.2	147.9±17.1	135.7±12.4
	well-differentiated	7	36.4±5.8	12.9±3.9	30.5±5.6	57.9±11.5	224.7±51.8	173.5±31.2	153.7±22.7

Note. N, number of patients; $p<0.05$ compared to the corresponding indices in $^*T_2$ and $^+T_{2-4}N_0$ groups.

in unchanged tissue, respectively. Probably, replacement of constitutive proteasomal subunits with immune ones potentiates enzyme activity of proteasomes [4]. This potentiation can also result from elevation of PA28 activator content.

Analysis of interrelationship between the examined parameters and clinical morphological indices of the disease showed that tumor size correlated with the content of PA28 complex (Table 1). In patients with tumor at T_{3-4} stages, the content of PA28 was higher by 31% than in patients with T_2 tumor.

In patients with regional metastases at stages $T_{2-4}N_{1-3}$, activity of 26S proteasomes was 1.7-fold lower than in metastasis-free patients with $T_{2-4}N_0$ tumors. Therefore, dissemination of the process is related to 26S proteasome pool. In patients with regional metastases, proteasomal composition was also changed: the content of immune LMP7 subunits increased by 107.4% compared to that in patients with $T_{2-4}N_0$ tumors. The formation of lymphogenous metastases probably modifies activity of 26S proteasomes in the primary tumor and elevates the content of LMP7 immune subunit. It can be somewhat related to manifestations of the non-immune functions by the immune proteasomes [6,10,12]. In addition, these changes can affect the amount of antigenic determinants in the primary tumor during regional tumor dissemination. We revealed no relations between the examined indices and the degree of tumor differentiation.

Thus, total activity of proteasomes and activity of 20S proteasomes were elevated in HNSCC tissue in comparison with correspondent indices in adjacent unchanged tissue. Activity of 26S proteasomes in the tumor correlates with regional tumor dissemination; hence, this parameter can be considered as a potential factor of disease prognosis. The growth and dissemi-

Fig. 2. Content of proteasomal subunits in tumor tissue during HNSCC in percentage to the corresponding values in unchanged tissue. The content of proteasomal subunits in unchanged adjacent tissue is taken as 100%.

nation of the tumor were accompanied by changes in subunit composition of proteasomes.

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