BIOGERONTOLOGY

Tumor-Modifying Effect of Cardiogen Peptide on M-1 Sarcoma in Senescent Rats

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The tumor-modifying effect of cardiogen peptide was studied on rats with transplanted M-1 sarcoma. The level of apoptosis of tumor cells after cardiogen injections in all experimental groups was higher than in the control. The dose-dependent inhibition of M-1 sarcoma growth after injection of cardiogen was caused by the development of hemorrhagic necrosis and stimulation of tumor cell apoptosis. The parameters of proliferative activity indicate that inhibition of tumor growth was not caused by the direct cytostatic effect of the drug on the tumor. Morphological signs indicate a specific mechanism of cardiogen action, realized through the vascular network of the tumor.

Key Words: *M-1 sarcoma; cardiogen; old rats*

Many factors affect the relationships between the organism and tumor in elderly and senile patients: specific properties of tumor cells, their microenvironment, components of nonspecific and specific resistance, cellular and humoral immune reactions to tumor cells [1-3]. The important role of cell-cell relationships in the mechanisms of oncogenesis should be emphasized, because these relationships integrate the systemic response of the organism to the development of malignant tumors [3].

The use of biostimulants improving immune defense and stimulating or not preventing the effects of antitumor drugs is a prospective trend in oncology [4,5-7]. Previous comprehensive morphofunctional analysis showed that cardiogen peptide stabilizes total homeostasis, stimulates cellular and humoral immunity, accelerated recovery of microcirculatory disorders, and regulates cell-cell interactions [2].

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We studied cardiogen peptide effects on M-1 sarcoma in old animals in order to evaluate the prospects of these peptides as tumor-modifying agents.

MATERIALS AND METHODS

The study was carried out on 24-month-old male outbred albino rats (180-200 g). The animals were kept at natural illumination on balanced rations in spring. The object of the study was M-1 sarcoma, a rapidly growing connective tissue tumor. The material for implantation was obtained from the tumor of a donor animal. In order to obtain a homogeneous tumor strain in the studied groups, the material from the same tumor was injected to 78 animals. After transplantation the animals were randomized into 5 groups. Group 1 (controls) comprised animals with implanted M-1 sarcoma (n=18). Group 2 animals received cardiogen on days 1-10 of sarcoma growth in a daily dose of 0.5 μg (10 injections every 24 h; total dose per rat was 5 μg). Group 3 animals received 10 injections of cardiogen on days 12-21 of tumor growth in the same

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dose according to the same protocol. Group 4 animals were injected with cardiogen on days 1-10 in a single dose of 5 μg (5 injections every 48 h, total dose per rat 25 μg). Group 5 animals received 5 injections of cardiogen on days 12-21 of tumor growth in the same dose according to the same protocol.

Tumor size at the site of transplantation was measured 2-3 times weekly and the volume was calculated by the formula for an ellipsoid:

$$V=4/3\pi\times(L/2)\times(D/2)^2,$$

where L is tumor length; $D=(D_1+D_2)/2$, where D_1 and D_2 are two perpendicular diameters. The term for histological study of the sarcoma in the experimental groups was determined by the dynamics and divergence of tumor growth lines. In the control group, the material for analysis was collected on days 16 and 26 of tumor growth; in groups 2 and 4 it was collected 12 days after the end of cardiogen treatment; and in groups 3 and 5 the material was collected 1 day after the last injection of cardiogen.

Sarcoma tissue was cut out in the form of 3-4-mm plates oriented along the long axis of tumor node with the adjacent skin (if possible) and fixed for 24 h in Bouin acid fluid for light microscopy and after Karnovskii for electron microscopy. Ultrathin sections contrasted with uranyl acetate and lead citrate, were examined under a JEM-100S electron microscope (JEOL). Common histology of the tumors was studied on sections stained with hematoxylin and eosin, toluidine blue, and by van Gieson method. Proliferating cells were immunostained using mouse monoclonal antibodies to proliferating cell nuclear antigen (PCNA; clone PC10, Calbiochem) and avidin-biotin-peroxidase kit for detection of mouse immunoglobulins (Vectastain). Apoptosis index (I_{AP}) and mitotic index (I_{MIT}) were evaluated by the standard method at immersion magnification; at least 3000 tumor cell nuclei were analyzed. The PCNA proliferative index (I_{PCNA}) was estimated as the proportion of quantitative density of PCNA-positive nuclei (N_{PCNA}) to quantitative density of tumor cell nuclei (N_{TCN}), stained with hematoxylin. Quantitative density was evaluated by the number of nuclear sections per mm^2 of section area. Evaluation of N_{PCNA}, N_{TCN}, and parameters of nucleolar organizer regions (S_{NO}, the mean section area of the nucleolar organizer region; ρ_{NO}, their volume percentage in the nucleus) was carried out by the IMSTAR computer analysis of microscopic images using Morphostar-2 and Colquant-2 applied licensed software in accordance with the basic stereology principles in morphometry [2].

The target structures were counted for each animal in 60 visual test fields by 3 sections of each analyzed tumor. The total test area for each tumor was at least 1.5 mm^2.

Parameters of tumor growth in the control and experimental groups were evaluated in 10 animals. The growth kinetics was calculated by the trend line equations for sites corresponding to certain stages of M-1 sarcoma development. In order to evaluate the tumor growth increment rate for the t_1-t_n interval, the geometric mean was calculated

$$G_{(t1\text{-}tn)}=\sqrt[n]{V(t_1)\times V(t_2)\times\ldots V(t_n)},$$

where $V(t_1)$ is tumor volume for t_1 term. The results were statistically processed using nonparametric Mann—Whitney *U* test.

RESULTS

In the control group, tumor nodules emerged under the hip skin on days 5-7 after transplantation. The tumors developed in all controls and by day 10 reached measurable size. The tumor growth was most intensive until day 24. The mean lifespan of rats after tumor transplantation was 37.4 days.

During the exponential growth phase M-1 sarcoma was presented by spindle and polymorphic cells with tissue and cell atypia. A characteristic feature of the M-1 sarcoma architectonics is regional heterogeneity of the vascular network development. The most active pronounced angiogenesis zone of M-1 sarcoma is its subcapsular region. The microcirculatory network in this zone was presented by thin-wall capillaries and sinusoidal vessels. Their endothelial wall consisted of a layer of elongated polygonal cells with uneven twisted borderline, tightly sticking to each other. The stroma was very scanty. Electron microscopy showed few fibroblasts along the vessels, solitary macrophages, and mast cells. Compact fine fibrillar basal membrane was seen under the endothelium. The vessels were surrounded by pericytes and an amorphous layer of intercellular matrix.

The M-1 sarcoma is characterized by pronounced regional heterogeneity of quantitative density of apoptosis distribution. The peripheral zone of tumors of 0.5-1.0 cm^3 volume contained 9-15 apoptotic cells per mm^2 of parenchymal area ($I_{AP}\approx0.3$). Up to 40-50 apoptotic structures, sometimes concentrated in small groups, were could be seen in the control test areas in the central regions of the tumor. A total of 3900-4300 tumor cells per mm^2 were detected in the subcapsular zone during the exponential phase of sarcoma growth. The I_{PCNA} varied from 70 to 90%. However, in some regions of the solid structure it decreased to 40-50%. The mitotic index was 1.9-2.6%, apoptosis index 0.2-0.4%.

The effect of cardiogen in a dose of 0.5 μg was studied during the latent period and exponential phase

of tumor growth (groups 2 and 3). Comparative analysis showed that the sarcoma growth curves in 8 animals from group 2 were below the tumor growth curve for the control group. According to coefficient α, no appreciable changes in tumor growth rate were observed until day 24. It is worthy of note however, that the tumor weight increment decreased by 25% (by geometric mean) during this period and was 0.48±0.09 (p>0.05). The growth curves for controls and experimental group 2 differed by the means only 2 weeks after cardiogen was discontinued. According to the coefficient α of trend line equation, the tumor growth rate between days 28 and 40 was 1.7 times inhibited, while tumor weight increment (by geometric mean) reduced by 33.2%. The difference is significant in comparison with the control group at p=0.05. The mean lifespan of animals with tumors in group 2 was 40.5±3.4 days (p>0.5).

In group 3, the kinetic parameters of sarcoma growth virtually did not differ from the control. During the exponential phase variations in individual tumor growth and the growth curves plotted by the means virtually coincided. The coefficient determining the growth kinetics by the exponential relationship and the tumor weight increment did not differ from the parameters in the control group. A trend to inhibition of growth rate and tumor weight increment was observed during the next period: coefficient α decreased to 1.16, $G_{28\text{-}40}$=13.56±1.63, the difference in comparison with the control being negligible. The mean lifespan of animals with tumors in group 3 reached 42.3±3.6 days (p>0.5).

Quantitative density of tumor cells in the subcapsular zone was 4080±190/mm^2 in group 2, 4136±170 in group 3. Proliferative activity (I_{PCNA}) remained as high as in the control group. On the other hand, according to computer analysis, index of apoptosis of tumor cells increased to 0.33±0.02 in group 2 and to 0.38±0.02 in group 3.

The effect of cardiogen in a dose of 5 μg was studied during the latent period and the exponential phase of tumor growth (groups 4 and 5). In group 4, the latent period before the appearance of tumor nodules was 6-7 days, but observations showed that only 80% transplanted cells survived. According to quantitative analysis, the increment of developing tumor weights during the exponential phase was 1.7 times higher compared to the control (geometric mean $G_{10\text{-}24}$=1.08±0.17), while on days 28-40 this value surpassed the control level by 23% ($G_{28\text{-}40}$=18.05±2.50). The mean lifespan of animals after tumor transplantation in group 4 was 35.9±4.2 days (p>0.5).

In group 5 rats no tumors developed. In 3 cases, the growth of transplanted tumors ceased after 2 injections of cardiogen in doses of 5 μg. These tumors completely regressed several days later. No appreciable deviations in the growth patterns of individual tumors were detected on days 10-24 in comparison with the mean values in the control group. The range of variations and growth curves, plotted by the means, coincided in general. However, on days 5-7 after the end of cardiogen injections, the growth curves in 4 animals started to shift from the means, characteristic of control animals. Inhibition of tumor growth rate persisted during about 10 days. According to the trend line equation, the coefficient α decreased by 20% from day 28 to day 40, while tumor weight increment was inhibited by 23%. The mean lifespan of group 5 animals was 38.9±2.6 days.

Study of the histological structure of tumors in groups 4 and 5 showed characteristic changes in the microcirculatory network in the capsule and subcapsular zone. The vascular lumen was dilated. Characteristic signs of edema with release of blood cells into the interstitium were seen perivascularly. Perivascular and interstitial edema was more pronounced in group 5. The results of analysis of histological preparations suggest that injections of cardiogen in rather high doses stimulated the hemodynamics in the tumors. This leads to increase of blood content in virtually all components of the vascular network in sarcoma during the early period, while later angiogenesis is stimulated. Proliferating tumor cells were concentrically grouped around the new capillaries, forming small groups. By their ultrastructural characteristics (large nuclei with hypertrophic nucleoli; cytoplasm saturated with free ribosomes, poorly developed Golgi complex), sarcoma cells in groups 4 and 5 virtually did not differ from tumor cells in the control group. Mast cells and monocytes in zones of their differentiation closely contacted with tumor cells. The mitotic, proliferative, and functional activities of endothelial cells were elevated in animals treated with cardiogen in a single dose of 5 μg. In addition, quantitative analysis detected a slight trend to intensification of the proliferative and functional activities of tumor cells in the subcapsular zone. On the other hand, tumor cell apoptosis increased. In group 4 the apoptosis index was 0.43±0.07, in group 5 0.49±0.02 (p<0.05).

The results of kinetic and morphofunctional studies indicate ambiguous effects of cardiogen on the growth of implanted connective tissue tumor. Its effect depends on its dose and stage of tumor development. Objectively recordable effect of tumor growth inhibition manifested only after injection of this drug in rather low doses during the latent period of tumor formation. Drug injections in the same doses during the phase of active growth of the tumor virtually did not modify its kinetic parameters. Increase of the single dose of the studied drug to 5 μg can lead to stimulation and to inversion of the inhibitory effect. The results of morphofunctional analysis indicate that

after prolonged treatment with cardiogen in high doses the main events developed in the perivascular stroma and were associated with angiogenesis stimulation. The maintenance of cell balance in the tumor under these conditions will be presumably determined by the proportion of intense proliferation and induced death of tumor cells. The results of quantitative analysis indicate that the level of tumor cell apoptosis after injections of cardiogen was higher in all experimental groups than in the control group.

These data suggest that further studies of cardiogen peptide as a presumable tumor-modifying agent are a prospective trend.

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