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MORPHOLOGICAL FEATURES OF DOXORUBICIN-RESISTANT WALKER 256 CARCINOSARCOMA AND RESPONSE OF MAST CELLS

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***Background*: The mechanisms of drug resistance of cancer have not been yet elucidated in details. Recently, the role of mast cells (MCs) in the development of drug resistance has been brought in the limelight. *The aim* of the study was to examine the morphological features of doxorubicin (DOX)-resistant Walker 256 carcinosarcoma and to assess the response of MCs and histamine content in these cells in relation to the development of resistance to DOX as well as in DOX-resistant tumors. *Materials and Methods*: The DOX resistance was induced by serial passages of Walker 256 carcinosarcoma in rats in the setting of DOX treatment *in vivo*. MCs in tumors were detected in the sections by staining with Toluidine Blue O. Histamine content in MCs stained with solution of Water Blue-Orcein was assessed by Astaldi semiquantitative method taking into account different staining intensity. *Results*: Formation of DOX resistance in the course of serial passages of Walker 256 carcinosarcoma was accompanied by the increase in the number of MCs in tumors and histamine content. Nevertheless, in tumors with phenotype of complete DOX resistance the number of histamine-containing MCs decreased to the same level as in tumors of the original strain that are DOX-sensitive. *Conclusion*: MCs are involved in formation of DOX resistance in Walker 256 carcinosarcoma.**

***Key Words:* mast cells, drug resistance, doxorubicin, histamine.**

Cancer drug resistance is one of the main factors limiting effectiveness of antineoplastic therapy. The mechanisms of both primary resistance featuring the intrinsic properties of cancer cells and acquired resistance arising as an adaptive response to chemotherapeutics of various groups are in the spotlight. Nevertheless, the mechanisms involved in the development of the resistance to anthracyclines have not been studied in depth.

Besides, in recent years, interest in properties of the mast cells (MCs) has increased due to their multifunctionality and involvement in adaptive responses and pathological processes. MCs population is heterogeneous. According to their protease content, human MCs have been divided into two phenotypes: those containing only tryptase, termed MC_T, and those containing both tryptase and chymase, termed MC_{TC}. MC_T are found in mucosa while MC_{TC} are considered as MCs of connective tissue type [1]. MCs are heterogeneous by many other factors: localizations, content of granules, response to different stimulation and pharmacological agents [2]. Nevertheless, such classification is rather conventional since MCs can dynamically change their properties according to the conditions of microenvironment [1]. On their surface, MCs express receptors to chemokines, immunoglobulins (IgA, IgE, IgG), adrenaline, adenosine, estrogen, leptin, histamine, serotonin, stem cell factor, etc [2].

MCs are capable of affecting tumor development, angiogenesis, and adaptive immune reactions [3, 4]. Due to proteases, MCs are involved in stroma remodelling, which promotes invasion and metastasizing of tumor cells [5, 6]. It is shown that density of MCs increases with tumor progression [7]. Nevertheless, there is no consistent view on the mechanisms of how MCs affect tumor growth. Two hypotheses of interactions between tumor and MCs exist. According to the first hypothesis, MCs stimulate carcinogenesis due to the expression of proteases, angiogenic and growth factors. According to the second hypothesis, MCs, on the contrary, possess cancer suppressive properties. The biological effects of MCs are mediated by the range of substances released by these cells. Therefore, the net effect of MCs on tumor growth depends on the complex interactions between these substances and stroma cells (endotheliocytes and fibroblasts) [8]. The heterogeneous phenotype both of cancer cells and MCs as well as the opposite effects of various factors in such interplay should also be taken into account.

Histamine is one of the mediators involved in regulation of wide range of physiological and pathological processes in which MCs are the main factors. This low-molecular monoamine participates in cell proliferation and differentiation, hematopoiesis, regeneration, wound healing, signal transduction in aminergic neurons, and also in a number of brain functions, in inflammation and modulation of the immune response [9].

Histamine plays an important role in physiological and pathological processes in mammary glands being involved in growth regulation, differentiation and function in pregnancy and lactation [10]. Four subtypes of histamine receptors (H_1, H_2, H_3 and H_4) have been revealed in mammary glands. Activation of H_2 and H_3 receptors promotes proliferation of cancer cells *in vitro*, while activation of H_1 and H_4 receptors inhibits such proliferation [11]. Also, histamine may affect tumor indirectly via activation of angiogenesis [12]. Histamine possesses both proangiogenic and antian-

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Abbreviations used: DOX – doxorubicin; MCs – mast cells.

giogenic properties depending on its concentration, availability of cofactors and tumor microenvironment. Nevertheless, histamine seems to affect angiogenesis not directly but via endothelial cells in cooperation with other proangiogenic factors.

The role of histamine in metastasizing is well known. The expression of MMR-2 and MMR-9 in breast cancer cells *in vitro* varies depending on histamine concentration, this effect being mediated by H_2 and H_4 [13]. Due to H_1 activation, histamine modulates MMR-2 activity not only in cancer cells but also in fibroblasts [14].

The role of MCs in the development of resistance to chemotherapeutic agents has not yet been clarified. Study of the factors triggering doxorubicin (DOX) resistance suggests that tumor cell-to-extracellular matrix interactions are important. The purpose of the study was to examine the morphological features of DOX-resistant Walker 256 carcinosarcoma and to assess the response of MCs and histamine content in these cells in relation to the development of DOX resistance.

MATERIALS AND METHODS

The rats from the animal facility of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, the National Academy of Sciences of Ukraine (Kyiv, Ukraine) were used in the study. The use and care of the experimental animals was performed in accordance with the standard international rules of biologic ethics and was approved by Institutional Animal Care and Use Committee. Walker 256 carcinosarcoma was obtained from National Repository of Cell Lines and Transplanted Tumors of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology. The rats were inoculated with original Walker 256 tumor cells or cells of DOX-resistant strain. Walker 256 carcinosarcoma with induced resistance to DOX was obtained as described earlier by serial transplantation of tumor cells in DOX-treated animals (12 *in vivo* passages in total) [15, 16]. The tumors of the parental strain, the tumors after four *in vivo* passages in DOX setting (partial DOX resistance), and the tumors of the refractory phenotype (complete DOX resistance) were studied. The separate group of the rats bearing Walker 256 carcinosarcoma of the refractory phenotype was treated with DOX at a dose of 1.5 mg/kg (accumulated dose of 7.5 mg/kg), and such tumors were also evaluated.

MCs in tumors were detected by staining with 1% solution of Toluidine Blue O, (Sigma-Aldrich, USA) in 0.5 M HCl by the standard technique [17] and counted as per 1 mm^2 of tumor tissue [18].

Histamine in MCs was detected in the sections stained with solution of Water Blue — Orcein [19]. The results of cytochemical reaction were expressed as a percentage of positive cells [20]. Histamine content in MCs was assessed by Astaldi semiquantitative method, taking into account different staining intensity (weak, moderate or intensive) in 100 cells.

The statistical analysis of data was carried out by method of variation statistics using Microsoft Excel 2010 (Microsoft Corp., USA). The arithmetic mean and its error (M ± m) were calculated. The statistical significances of differences between mean values were assessed with Student's *t*-test. Differences at $p < 0.05$ were considered significant.

RESULTS

Morphological features of Walker 256 carcinosarcoma. In 6 days after transplantation of parental strain of Walker 256 carcinosarcoma, tumor node of 2.3 ± 0.3 cm was evident. Tumor cells were bunched in bundles and layers or solid complexes plunged into muscular tissue here and there and sprouted along muscle fibers and blood vessels disarranging the structural architectonics of skeletal muscles. Sarcomatous cells with hyperchromic nuclei varied in form (spindle, stellated) and size. Epithelioid cells were distinguished by moderate polymorphism and had mainly hypochromic nuclei. In these cells, individual mitoses have been observed. On the periphery of tumor node, epithelioid cells formed so-called pseudo-follicular structures and solid layers. The tumors were, as a rule, limited to the surrounding sarcomatous stroma (Fig. 1). At the center of the tumor, the tissue was less structured than on the periphery, polymorphism of cells and nuclei was less expressed. Necrotic foci of various sizes were observed.

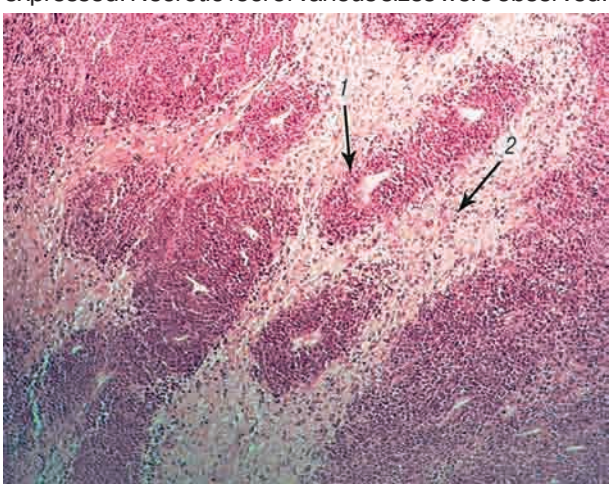

Fig. 1. Walker 256 carcinosarcoma: pseudo-follicular structures (1) and solid bundles (2) (stained with hematoxylin and eosin, x 200)

In 16–18 days after transplantation, the tumor node took on more structured patterns with formation of pseudo-follicular structures and outgrowth of the bundles of sarcomatous cells with light epithelioid cells located between them. At sites of proliferation, active angiogenesis with ingrowth of blood vessels into the tumor has been observed. The remaining muscle fibers were lysed. In the center of tumor node, necrotic foci have been observed. The increased number of apoptotic cells has been evident.

MCs in Walker 256 carcinosarcoma tumors. A small number of MCs was detected at the central sites of the tumors (6.7 ± 1.3 cells per 1 mm^2), some of them with metachromatically stained granularity; some grains sticking together (Fig. 2). Most of these cells were destroyed partially or completely. In MCs that seem to be intact, specific granules exited outside.

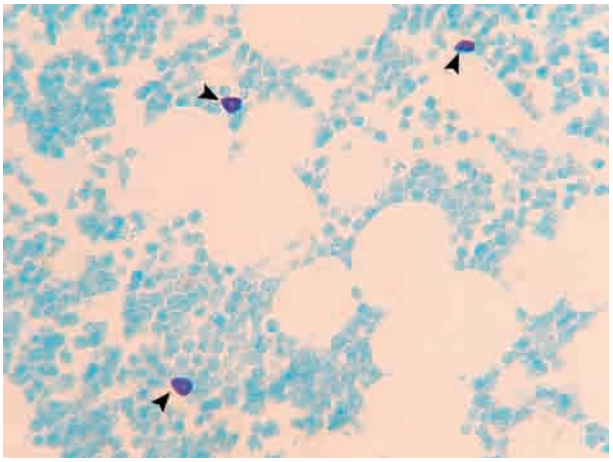

Fig. 2. MCs in a section of Walker 256 carcinosarcoma (control) (stained with Toluidine Blue O, × 400)

It is necessary to notice that MC count (up to 15–20 per 1 mm^2) increased when the inflammation foci were observed. In such cases, MCs were larger and contained specific metachromatic granularity in a cytoplasm. Observed degranulation of MCs with the release of the specific granules seems to indicate their functional activity. Such cells were found mainly at the peripheral sites of tumor node.

Morphological changes of Walker 256 carcinosarcoma throughout formation of DOX resistance and in DOX-resistant tumors upon DOX treatment. In tumors with partial DOX resistance (four *in vivo* passages in DOX setting), tumor cells were less polymorphic. Light epithelial cells in clusters prevailed, in particular, on the periphery of tumor node. At the central sites of tumor, cells with moderate nuclear polymorphism were predominant along with the necrotic foci. The number of blood vessels of capillary type increased. Also, in comparison with original tumors, partially resistant tumors were characterized by more structured patterns with formation of pseudofollicular structures and the cords of sarcomatous cells with dark hyperchromatic nuclei.

In tumors with complete DOX resistance, vascularization further enhanced, mainly at the expense of the vessels of capillary type with increasing hemorrhages accompanying with infiltration and plasmatic suffusion of the tumor. In the central zones of the tumor, the necrotic foci of various size, mainly infiltrated with neutrophils and monocytes were observed. On the periphery, tumor cells were bunched in bundles and layers of solid complexes that plunged into muscular tissue, while the remnants of muscles were also evident (Fig. 3). Epithelioid cells of moderate polymorphism and with prevalence of hypochromic nuclei prevailed and formed pseudofollicular structures and solid layers with bundles of sarcomatous cells. The number of mitoses in these cells increased. The pathological mitoses were also evident.

DOX treatment of the rats bearing DOX-resistant Walker 256 tumors has not changed substantially the morphology of tumors. Epithelioid cells remained prevalent but fibrotization increased in comparison with resistant type (Fig. 4). Furthermore, the significant vascularization mainly at the expense of capillary vessels and increasing numbers of hemorrhages was evident.

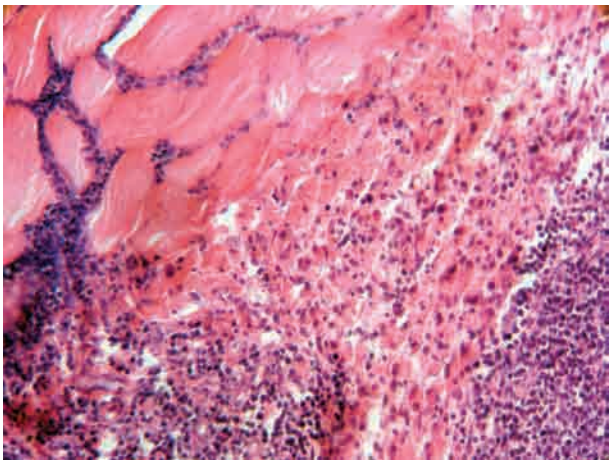

Fig. 3. The cords of Walker 256 carcinosarcoma DOX-resistant tumor cells growing into muscular tissue (stained with hematoxylin and eosin, × 400)

Thus, with increasing DOX resistance of Walker 256 carcinosarcoma the number of capillary vessels increased, epithelioid cells being the main component of tumors. The fibrotization of tumor node was also observed. Upon DOX treatment of DOX-resistant type of Walker 256 carcinosarcoma, the features above became more prominent.

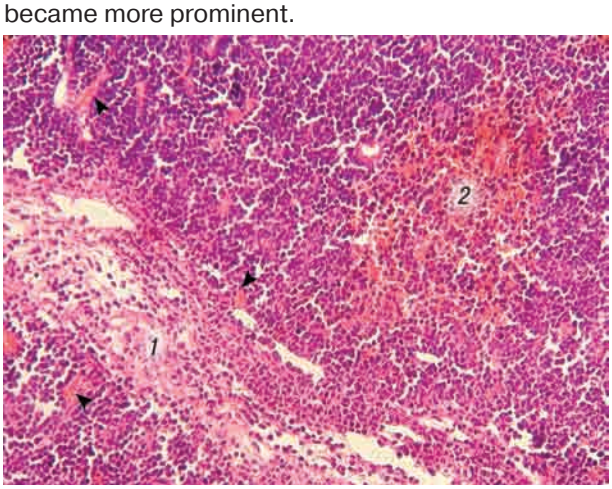

Fig. 4. Elements of connective tissue (1), hemorrhages (2), vessels (arrows) in DOX-resistant Walker 256 carcinosarcoma upon DOX treatment (stained with hematoxylin and eosin, × 400)

MCs in Walker 256 carcinosarcoma throughout formation of DOX resistance and in DOX-resistant tumors upon DOX treatment. In tumors with partial DOX resistance, MCs located mainly in connective tissue layers. Occasionally, MCs were observed near blood vessels. Granules in cytoplasm did not stick together suggesting MC metabolical activation. Some granules were found extracellularly while MCs remain intact.

In tumors with complete DOX resistance, MCs located centrally or among pseudo-follicular structures, or near blood vessels. The insignificant part of such cells was degranulated with the granules exiting the cells while a fraction of granules remaining in a cy-

toplasm (Fig. 5). MCs were also found in connective tissue structures surrounding tumor node.

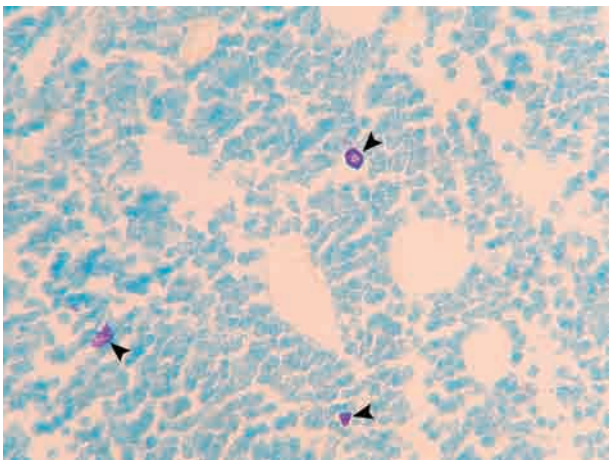

Fig. 5. MCs (arrows) in central zones of DOX-resistant Walker 256 carcinosarcoma (stained with Toluidine Blue O, × 400)

After DOX treatment, the number of MCs in DOX-resistant tumors increased compared to that in DOX-resistant tumors of animals which were not DOX treated (Fig. 6). These cells localized predominantly in the connective tissue components of tumors and near the vessels of capillary type crowded with erythrocytes. In cytoplasm of the most part of these MCs, disperse granules were visible with increased number of the degranulated cells wherein specific granules being released extracellularly.

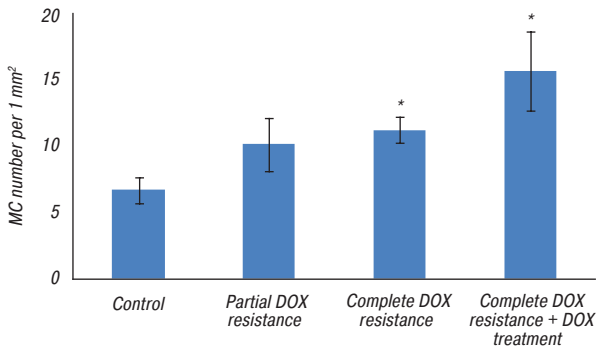

Fig. 6. The number of MCs in DOX-resistant Walker 256 carcinosarcoma in animals treated or non-treated with DOX. *$p \leqslant 0.05$ comparing with control

Histamine content in MCs in Walker 256 carcinosarcoma throughout formation of DOX resistance and in DOX-resistant tumors upon DOX treatment. In animals bearing original Walker 256 tumor, histamine-containing MCs were observed mainly near blood vessels, on the periphery of tumor node, and also at the sites where tumor cords grew into the muscles (Fig. 7). In partially resistant tumors, the number of histamine-containing MCs increased significantly (Fig. 8, 9) while the count of MCs in completely resistant tumors dropped to the initial level in the original tumor (Fig. 9). DOX treatment of animals with DOX-resistant tumor did not further affect histamine-containing MC count.

The percentage of histamine-positive MCs with intensive staining increased with increasing DOX resistance with the concomitant decrease in the number of moderate and poorly stained histamine-positive MCs (Fig. 10).

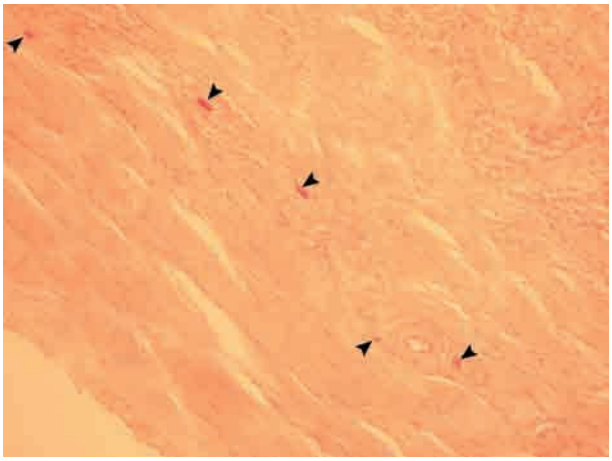

Fig. 7. MCs (arrows) at the site of tumor ingrowth into muscular tissue (stained with Water Blue — Orcein, × 200)

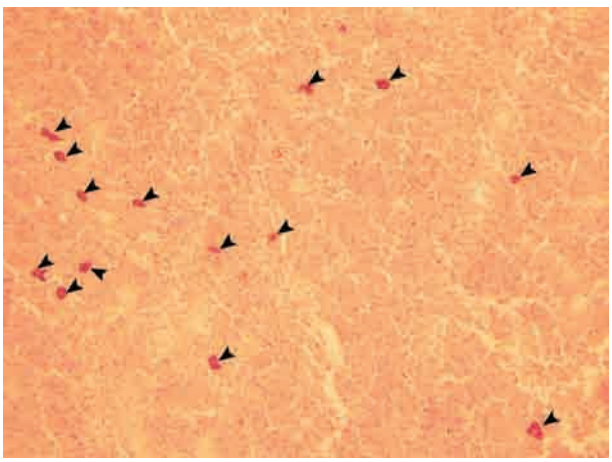

Fig. 8. MCs (arrows) in central zones of Walker 256 carcinosarcoma with phenotype of partial resistance (stained with Water Blue — Orcein, × 200)

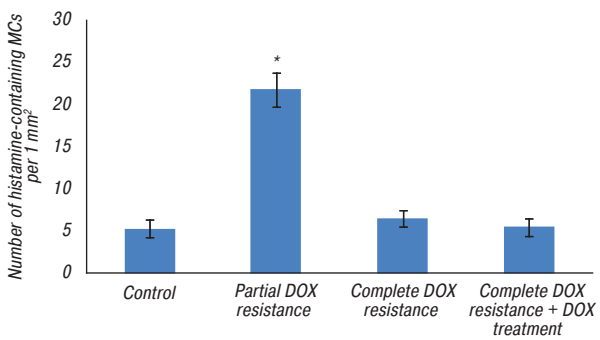

Fig. 9. The number of histamine-containing MCs in DOX-resistant Walker 256 carcinosarcoma in animals treated or non-treated with DOX. *$p \leqslant 0.05$ comparing with control

DISCUSSION

As was shown earlier, the formation of complete DOX resistance of Walker 56 carcinosarcoma in rats requires 12 passages in the setting of DOX treatment *in vivo* [15]. Four courses of chemotherapy result in partial DOX resistance with tumor growth inhibition by about 30%, while DOX treatment inhibited the growth of parental Walker-256 carcinosarcoma by about 65% [16]. These two time-points corresponding to partial (4 passages) and complete (12 passages) DOX resistance were selected for studying the morphological features of Walker 256 carcinosarcoma

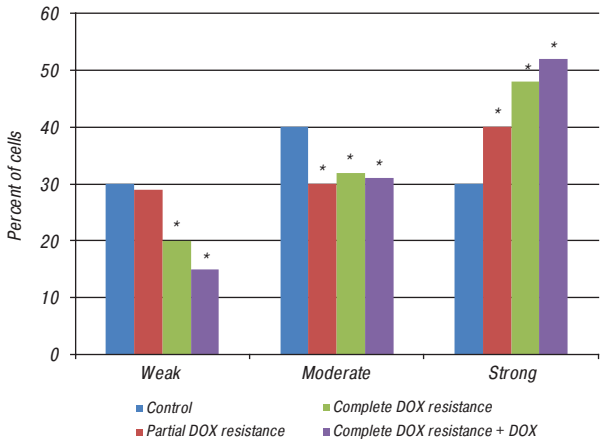

Fig. 10. Distribution of histamine-containing cells by staining intensity.*$p \leqslant 0.05$ comparing with control

and the biology of MCs in dynamics of the formation of DOX resistance.

Morphologically, in DOX-resistant tumors we have found more structured patterns with formation of pseudo-follicular structures, slight outgrowth of the bundles of sarcomatous cells, and the activation of neoangiogenesis. MCs response was also evident with the increasing functional activity of these cells manifested as their degranulation with the release of the specific granules. The data obtained correspond to the results of other researchers who showed the increased MC count, basically, at the peripheral sites of a tumor and surrounding tissues [21, 22] with degranulation of single MCs in the centre of tumors [23]. The slight increase in the number of these cells with small content of intracytoplasmic granules was observable mainly near small vessels.

In DOX-resistant tumors, the outgrowth of connective tissue component with fibrotization of tumor node was noted. This fact can be explained by MC contribution to remodeling of connective tissue structures due to the activity of the matrix proteinases and proteinases located in MC granules as well as the intensification of collagen synthesis by activated fibroblasts [24]. The significant vascularization in DOX-resistant tumors seemed to be associated in part with known MC effects on angiogenesis due to histamine and VEGF [25, 26].

Two biologically active agents produced by MCs, heparin and histamine, acting oppositely play an important role in regulation of MC effects [27]. The antagonism of these substances is the cornerstone of the functional duality of MCs acting both in stimulation and inhibition mode.

The number of MCs in DOX-resistant tumors sequentially increased, and the number of MCs containing histamine decreased. It is known that DOX may stimulate secretion of histamine by MCs [28, 29] similarly to substance 48/80, which leads to degranulation of MCs. This occurs because heparin and chondroitin sulfate are protein glycanes with strong negative charge while DOX has a positive charge facilitating its binding with heparin and protein glycanes that, in turn, stimulates histamine release from MCs.

The phenotype of DOX resistance in Walker 256 carcinosarcoma is associated also with changes in chemical signals produced by the cells of resistant tumors. The mediators secreted by tumor cells may affect immunocompetent cells changing the patterns of their synthesis and secretion [30]. In this setting, the response of MCs is expressed not only as an increase in MC number but also in changing patterns of mediators located in cytoplasm of these cells (both preformed and newly synthesized). In particular, we have shown that histamine content in MCs upon DOX treatment decreased. But the decrease in histamine content does not imply the decrease in their functional activity. On the contrary, histamine is released from cytoplasmatic granules into extracellular space. The released histamine may enhance proliferation of tumor cells, stimulate angiogenesis, increase activity of matrix metalloproteinases (MMP-2 and MMP-9) and provide for immunomodulating effect on immune competent cells (macrophages, T-cells) [9, 13]. As to the stimulation of angiogenesis, we in fact demonstrated the increased density of capillaries in DOX-resistant tumors. Also, histamine is capable to increase expression of periostin and collagen I in fibroblasts [31] possibly contributing in the increased fibrotization of tumor node demonstrated in DOX-resistant tumors.

Our data are in line with the current opinion that MCs are important in regulating different functions relevant to pathogenesis of tumors and adaptation of tumor-bearing host to the extreme factors including chemotherapeutical agents. We have shown that MCs involved in extracellular matrix remodeling contribute to the formation of DOX resistance of Walker 256 carcinosarcoma.

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