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Histochemical comparative study of primary and metastatic tumors in nude rats xenotransplanted with KB cells

Estudo histoquímico comparativo da matriz extracelular de tumores primários e metastáticos em ratos nude xenotransplantados com células KB

Terezinha Regina Prúpere OGATA[1]; José Luiz GUERRA[2]; Francisco Javier Hernandez BLASQUEZ[3]

CORRESPONDENCE TO:
José Luiz Guerra
Departamento de Patologia
Faculdade de Medicina Veterinária e Zootecnia da USP
Av. Prof. Dr. Orlando Marques de Paiva, 87 - Cidade Universitária Armando Salles de Oliveira - 05508-900 - São Paulo SP - Brasil
e-mail: guerra@usp.br

1. Laboratório de Anatomia Patológica - Instituto Butantã - SP
2. Departamento de Patologia - Faculdade de Medicina Veterinária e Zootecnia da USP - SP
3. Faculdade de Zootecnia e Engenharia de Alimentos, Pirassununga, SP

SUMMARY

A comparative study of metastic and primitive tumors extra-celular matrix in nude rats, xenotransplanted with KB cells, using histochemical methods, was carried out. In both primitive and metastatic tumors it was possible to observe a qualitative and a quantitative variation of matricial components; coexistence of different kinds of fibers; small representation of elastic fibers; presence of acid and sulfated glycosaminoglycans and neutral polysacharyds, and also absence of basal membrane. It is suggested a small interaction between collagenic components and proteoglycan of primary tumors and the proteoglycan, whereas in metastases this interaction is increased.

UNITERMS: Extracellular matrix; KB cells; Histochemical; Nude rats

INTRODUCTION

Metastasis are the main cause related to morbidity and mortality in patients with malignant tumors. Curent therapy modalities like surgery, chemoterapy and radiotherapy may heal about 50% of the patients who develop a malignant tumor. Failure in treatment, in most cases, is due to either direct effect of metastases, or to complications associated to their treatment.

As a consequence, cancer researchers greatest challenge has been to develop and improve methods which may predict metastatic tumors trends for each patient, clinically identifying an hidden micrometastases, preventing local invasiveness, and also treating them[15]. Great progress have already been accomplished, studying the interaction between metastatic cells and extra-cellular matrix. The latter segregates tissue components and keeeps the cells together, thus determining tissue architecture. It also acts as a macro-molecular filter; and influences morphological growth and cytodifferentiation[14]. However in neoplams, these interactions may be modified, influencing tumoral proliferation and invasiveness. Changes in the matrix may have several causes – action of degrading enzymes, defective basal organization, or its loss[20]. Because of the importance of the relationship between tumoral cells and extra-cellular matrix in metastization, it was investigated by histochemical methods in primary tumors as well as in metastatic ones. It was used nude rats as experimental models, inoculated with KB cells[4].

MATERIAL AND METHODS

ANIMALS: homozigotic (rnu/nu) nude rats, Rowett lineage, males and females, weighing from 7 to 9 grams, were used. Matrices came from CNRS – Centre National de la Recherche Scientifique, in France. To obtain metastases, animals are ideally two days old[7]. Animals used in these experiments were kept in special animal housing at Butantan Institute – São Paulo – Brasil.

EXPERIMENTAL TUMOR: KB cellular lineage from human oral cavity squamous cell carcinoma, kept in permanent culture since 1954[8] was used. Cells were acquired from Adolpho Lutz Institute – São Paulo – Brasil. The first inoculation was perfomed in adult animals using $1x10^6$ cells/ml inoculated in the axilar region[4,13]. Further inoculations were performed through serial passages, from one animal to the other. After excising tumoral mass and fragmenting in the Hank's medium, animals were anesthetized with ethilic ether vapour and received tumoral fragments of about 1 mm^3 each, subcutaneously, in the axilar region. They were observed daily, in order to follow tumoral mass growth.

MICROSCOPIC EXAMINATION OF PRIMARY TUMORS AND LUNG METASTASIS: sixty days after inoculation, animals were sacrified and necropsied. Representative fragments of primary and lung metastatic tumors were fixed in 10% formalin buffered with 1% calcium carbonate solution, embedded in paraffin and cuts 4 µm in thickness were obtained. Slides stained with hematoxilin-eosin (HE) plus histochemical methods for interstitial matrix. Cuttings of fragments and controls were 8 µm in thickness

each, and were submitted to following selective techniques:
- Picrosirius staining in association with polarization microscopy for colagenous fibers[8].
- Gordon; Sweat's method[6] for reticular fibers; Weigert's fuesin resorcin method[17] for elastic fibers; PAS technique in accordance to Lison[16] to detect neutral polysacharids; Lison's Alcian-blue[6] to determine acid polysacharids; Gomori-Grocott's method using metenamine silvery for basal membrane[17].

DETERMINATION OF COLLAGEN PROPORTION PRIMARY TUMORS AND IN LUNG METASTASIS: sections of 8 µm in thickness were stained the picrosirius technique. The area collagenous content in four slides was determined by direct measurement, with aid of DIRACOM 3 Program of Image Analyses System of Morphometric Analyses – of the Dentistry applied Data Laboratory (LIDO) in the Stomatology Departament of the School of Dentistry of the Universidade de São Paulo. Ninety areas of 596,475 μm^2 each were measured, fifty of them being tumoral and forty of them metastatic. The percentage of collagenous area, in relation to tumoral parenchyma, for both cases, was calculated as an estimate of the collagenous amount in tumor and metastasis. The medians of these areas were statistically compared using Student's T-test and variance analysis[2].

RESULTS

The macroscopic aspects of the tumoral masses in the implant areas was well-defined, characterized by delimitation and non-invasion of adjacent structure. Whitish tissue with large necrotic areas in the central portion was visible in cutting. Pulmonary metastases were diffusely scattered in the parenchyma of both lungs, and were characterized by well-delimited multiple nodules. In surface cuts, necrotic areas were seen.

Histological sections of primary tumors, stained by HE, demonstrated well-delimited epithelial neoplasia with intense cellular pleomorphism, many mitotic figures; delicate and rare stroma. Histological findings complete analyses led to the conclusion of squamous cell carcinoma, poorly differentiated. As for the histological sections of the lungs, also stained by HE, metastasis of squamous cell carcinoma poorly differentiated, constituted by intensely pleomorfic cells, moderate mitotic index and atypical mitoses were evidenced.

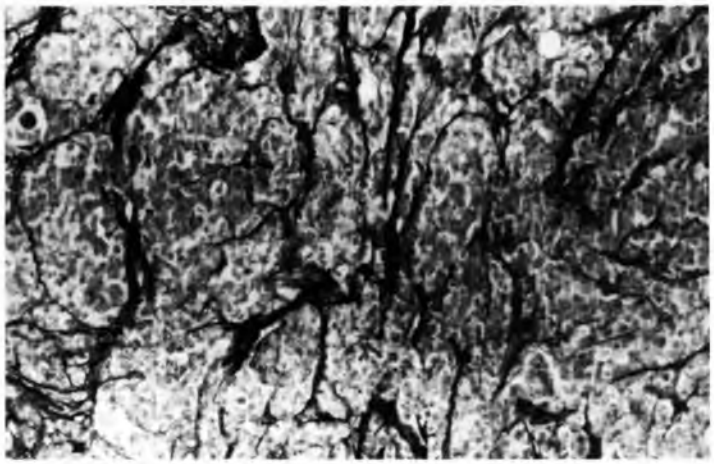

Figure 2
Histological aspects of argirofilic fibers primary tumor submitted to argentic impregnation (66x).

Study of the primary tumors by picrosirius method evidenced the presence of thick fibers of collagen, irregularly distributed in the parenchima together with a small amount of thinner fibers, irregular similary arranged as well (Fig. 1). When this technique was associated to polarization, it was possible to observe that collagen present was formed in most parts by thick fibers, yellowish or reddish, strongly birrefringent, suggesting type I collagen. Some fibers were thin, pale, greenish and weakly birrefringent, probably type III collagen. Argentic impregnation of histological sections showed a large amount of reticular fibers forming a supporting net for tumoral tissue (Fig. 2). Regarding the distribution of collagen in lung metastases, stained by picrosirius method, it could be observed the presence of thin fibers, regularly distri-buted, generating a lobular arrangement for the neoplasm (Fig. 3). There was still thick redish fibers, strongly birrefrigent, just as it may be seen in type I collagen. Argentic impregnation showed the presence of reticular fibers disposed in a way similar to that evidence by picrosirius method (Fig. 4).

Study of the elastic system by Weigert method allowen in

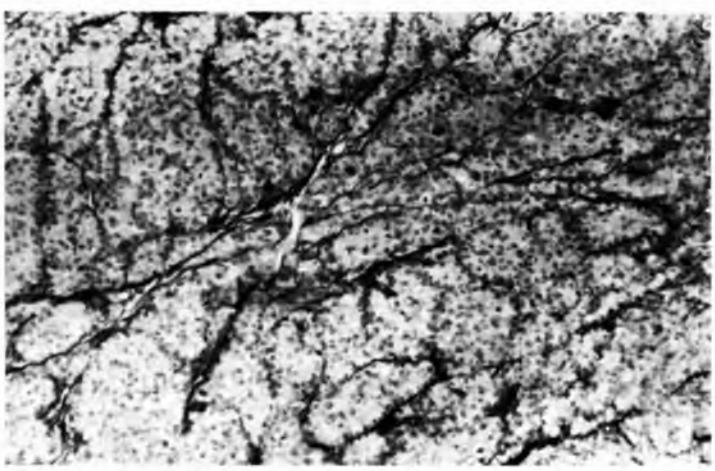

Figure 1
Histological aspects of collagen in primary tumor stained by Picrosirius (66x).

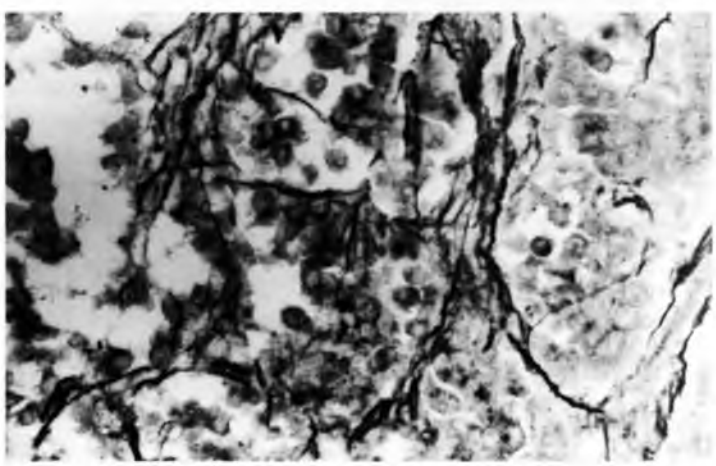

Figure 3
Histological aspects of collagen in lung metastasis stained by Picrosirius (66x).

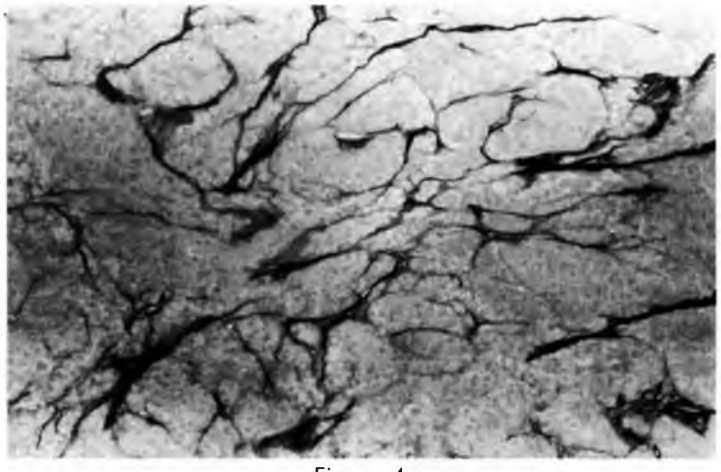

Figure 4
Histological aspects of argirofilic fibers in lung metastasis submitted to argentic impregnation (66x).

visualization of thin fibers delimiting small groups of tumoral cells in primary tumors and a similar finding could be observed in lung metastasis, although in smaller amounts.

The presence neutral polysacharyds in both primary tumors and metastasis has been observed using PAS. The same fact occurred in relation to the presence of acid and sulfated glycosaminoglycans in the stroma of both neoplasms, which was evidence by Alcian blue in pH 0,5. It was not possible to make these substances evident throught histochemical methods in the basal membrane.

Statistical analysis allowed the conclusion that medians of collagenous areas primary tumors and those in lung metastases differ significantly, being greater in primary tumors.

DISCUSSION

Analysis of literature clearly shows that neoplasms are quite well studied regarding their most varied aspects, though studies on extra-cellular matrix have been less frequent and less comprehensive, despite its relevance. The results found showed the variability of the extra-cellular matrix, when primary tumors of KB cells were compared to their lung metastases.

Quantatively, primary tumors had a larger amount of collagen than the metastases, which could be detected statistically. Qualitatively, though primary tumors presented collagen consisting mostly of fibers that seemed to be type I, whereas those from the lung metastases were more similar to reticular or type III fibers, what is in agreement with other kind of tumors described literature[10,12].

Likewise, the co-existence of different kinds of fibers in both loci was expected, since the occurrence of just one type of collagen in organs or tissues is quite rare. Furthermore, type III collagen is generally found where type I fibers also exist[3].

Based on the differences found among collagen types present in primitive and metastatic tumors, it seemed feasible to bring up the hypothesis that medium where tumors develop may be the main modulator for the synthesis of components of the neoplam extracellular matrix. This hypothesis was grounded on the existence of a large amount of reticular fibers or type III collagen in the stroma of normal lungs[1,18].

For the study of collagen in this experimental model, the utilization of the picrosirius method associated to polarization and to the argentic impregnation method, proved to be useful and sensitive in detecting those structures which are likely to have collagen molecules organized in fibriles and fibers, making it possible is use the technique in acquiring more knowledge collagenic components of the other neoplams[19]. Regarding elastic fibers, the most probable hypothesis is that they are not involved in the molecular basis of this neoplasm, if one bears in mind their small representativity in both primary tumors and metastasis.

Some authors[11] have demonstrated that the type of glycosamino-glycans found in a determined tissue is specifically related to the type of collagen present in the same tissue. It is known that heparan sulfate and dematan sulfate are acid and sulfated glycosaminoglycans, respectively, found in areas containing type III and I collagen.

Considering that for both tumors histochemical study for determination of glycosaminoglycans was positive and showed the same color intensity (alcian blue method pH 0,5 and pH 2,5), we concluded that, in their majority, they were sulfated.

Still in agreement with some authors[9], there was a correlation between the type of collagen found in a tissue and its degree of interaction with proteoglicans, which is maller in type I collagen and greater in type III colagen. Therefore, for the same reasons given above, it was suggested that interaction between fibers and proteoglicans (mainly with dermatan sulfate) in primary tumors is smaller, but it is greater in lung metastases (specially with heparan sulfate).

Regarding neutral polycacharyds, our results were positive for both tumors, possibly the presence of reticular fibers, though the amount of the fibers was greater in metastatic tumors.

It seems valid to ponder that the larger amount of reticular fibers in metastases is due to a smaller need of sustenance and defence of these foci in relation to primary tumors, which develop in cellular subcutaneous tissue.

Basal membrane was not evidence by usual histochemical methods. This suggests that it is related to the degree of invasiveness of the neoplasm. Opportunely, we intend to make it evident performing an ultrastructural study.

The results indicate that the site of tumoral growth modulates in some way the expression of the components of the extracellular matrix.

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RESUMO

Estudou-se comparativamente, através de métodos histoquímicos, a expressão dos componentes da matriz extracelular de tumores primitivos e metastáticos em ratos nude, xenotransplantados com células KB. Em ambas as neoplasias observou-se uma variabilidade tanto qualitativa como quantitativa dos componentes matriciais, coexistência de diferentes tipos de fibras, pouca representatividade de fibras elásticas de glicosaminoglicanas ácidas e sulfatadas e de polissacarídeos neutros, além da ausência de membrana basal.

UNITERMOS: Matriz extracelular; Células KB; Histoquímica; Ratos nude

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