LYMPHATIC DISSEMINATION IN NEOPLASIA: DETERMINATION OF NUCLEAR VOLUME AND DNA CONTENT OF PRIMITIVE AND REGIONAL LYMPH NODE EHRLICH TUMOR CELLS

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SUMMARY: When inoculated into the footpad of mice, Ehrlich tumor grows in a solid form and disseminates to popliteal lymph nodes. This study was performed in order to characterize the tumor cells that migrated from footpad to popliteal lymph nodes. The nuclear volume of primitive and lymph node tumor cells was measured with a caryometric ocular. DNA quantitation was performed in Feulgen stained slides by scanning cytophotometry. Tumor cells harvested from popliteal lymph nodes one hour after inoculation into the footpad showed no statistical differences in DNA quantitation from original ascitic and footpad tumor cells. Tumor cells in popliteal lymph nodes 30 days after inoculation showed a smaller nuclear volume, but presented the same DNA content as the cells that grew in the footpad. Tumor cells in the footpad 30 days after inoculation showed a greater DNA content than those in the footpad one hour after inoculation. These results suggest a possible selective effect for Ehrlich tumor cells when they grow in the footpad, but not when they metastasize to regional lymph node.

UNITERMS: Lymphatic metastasis; Carcinoma Ehrlich tumor; DNA

INTRODUCTION

Nowadays cancer is defined as a genetic disease caused by a number of known and unknown factors[19]. Once started, neoplastic progression is usually accompanied by increased genetic instability. This may generate a phenotypic diversity manifested by different tumor cell clones, as proposed by NOWELL [10] (1976) and PAGE et al. [12] (1988).

The biological behaviour of malignant neoplasms is ruled by their ability to metastasize. This characteristic makes the disease to develop and determines the evolution of the process [14]. Metastases are secondary tumor growths, and are reported to be responsible for the death of over 70% of the cancer patients [2].

Among the various routes by which tumor cells disseminate, the hematogenous and the lymphatic ones are the most common [13]. The initial mode of spread of many cancers is by lymphatics, this mechanism being poorly understood [16]. An intriguing "preference" for assuming a metastatic route and a target organ is also reported [20] for many different kinds of neoplasms.

The process of metastasis formation includes a sequence of steps during which tumor cells are challenged by several mechanisms as, for exemple, immunological ones [6,9]. Because of this, many evidences suggest that the inner characteristics of tumor cells determine their ability to metastasize [5,7].

We reasoned that the migration ability of tumor cells might be related to changes in their morphologic and caryotypic profiles, as a consequence of selection. Description and comparison of phenotypic and genotypic characteristics of primitive and migrating tumor cells may be of value to type the cells with metastatic potential.

The Ehrlich tumor, that grows in mice in both ascitic and solid (subcutaneous) forms, disseminates spontaneously to regional or draining lymph nodes. This transplantable tumor was used here as a model of lymphatic dissemination. The DNA content and the nuclear volume of primitive and lymph node tumor cells were determined.

MATERIAL AND METHOD

ANIMALS - Swiss male mice weighing 25-35 g, fed with balanced diet and water "ad libitum" were used.

TRANSPLANTABLE TUMOR - The Ehrlich tumor is kept in our laboratory by serial intraperitoneal transplantation performed in mice at 10 day intervals. Approximately 3 ml of ascitic fluid were collected from a donor mouse and

centrifuged at 1000 g for 3 minutes. The cells in the pellet were washed three times in phosphate buffered saline (PBS), resuspended in the same solution, and counted in order to standardize a concentration of 5×10^7 tumor cells/ml. To obtain a solid tumor, about 0.05 ml of this suspension, containing 2.5×10^6 cells, were carefully inoculated into the left footpad of mice.

EXPERIMENTAL PROCEDURES

DETERMINATION OF THE NUCLEAR VOLUME - Five mice were inoculated as described earlier. After 30 days, they were sacrificed by prolonged exposition to ether. Their left footpad were dissected, and samples of the growing tumors obtained from the site of inoculation, as well as the ipsilateral popliteal lymph nodes, were fixed in 10% buffered formalin. Five micrometers histological sections were cut from paraffin blocks and stained with hematoxilin and eosin (H.E.). The longest (LA) and the shortest (SA) nuclear axes of about 30 randomly selected tumor cells of each footpad and lymph node were measured in a Zeiss microscope, provided with a Zeiss caryometric ocular. These dimensions were converted into volume (cubic micrometers) using the equation described by VALERI [16] (1954).

DNA QUANTITATION - For this assay, 10 mice were inoculated into the left footpad as described earlier. One hour and 30 days after, groups of animals were sacrificed. Their left footpad and the ipsilateral popliteal lymph nodes were collected, macerated in a sterile PBS solution, filtrated and inoculated intraperitoneally in receptor mice. Approximately 15 days after, the animals developed tumor ascites. A drop of the ascitic fluid was spread on a glass slide, fixed for 3 minutes in methanol, and stained with the Feulgen's method. The quantitation of nuclear DNA was performed by scanning cytophotometry, as described by SOMA [15] (1981). The groups of ascitic tumors were designated as follows :

OT : original ascitic tumor

F1H and F30D : footpad tumor cells collected 1 hour and 30 days after inoculation respectively.

LN1H and LN30D : lymph node tumor cells collected 1 hour and 30 days after inoculation respectively.

The amount of DNA of about 35 cells of each group was determined in a Zeiss scanning cytophotometer, connected to a Polymax microcomputer. The obtained values were compared to those of the **Bufo ictericus** erythrocytes, and converted into picograms (pg).

STATISTICAL ANALYSIS - The significance of the results was verified by using Student's T test ($p < 0.05$). The analysis of variance (ANOVA), followed by Duncan's test, was applied to detect statistical differences between the groups of DNA quantitation.

RESULTS

The microscopic features of the footpad 30 days after the inoculation showed the presence of an exuberant tumor mass of pleomorphic, highly anaplastic cells invading the dermis. Some giant, multinucleated cells and many atypical mitotic figures could be seen. Necrosis occurred in extensive areas, and the survivor cells were localized almost exclusively around the blood vessels.

Most of the neoplastic cells in the popliteal lymph nodes were found in the subcapsular spaces. Sometimes they were seen in groups, as if growing in this region. A metastatic focus, distorting completely the lymph node architecture, was seen in one popliteal lymph node.

The mean values (and standard deviations) of the longest (LA), shortest (SA) axes and nuclear volume (NV) measurements are presented in Tab. 1. Tumor cells that migrated to regional lymph nodes showed a smaller nuclear volume than those that grew in the footpad ($p < 0.05$).

The results of DNA measurements are presented in Tab. 2. Tumor cells obtained from both footpad and popliteal lymph nodes one hour after inoculation (F1H and LN1H) showed comparable DNA content to the OT (original ascitic) cells ($p < 0.05$). Nevertheless, the mean of DNA content in tumor cells obtained from both footpad and popliteal lymph nodes 30 days after inoculation (F30D and LN30D) differed significantly from the OT, F1H and LN1H groups. No statistical differences were detected between LN1H and F1H groups and neither between F30D and LN30D groups.

DISCUSSION

Ehrlich tumor cells inoculated into the footpad and the subsequent analysis of the regional popliteal lymph nodes may be considered a suitable model for the study of lymphatic metastasis according to the criteria defined by VAN DE VELDE; CARR [17] (1977). A more detailed study on the lymphatic dissemination of the solid Ehrlich tumor in mice has been published [4].

This study has shown that Ehrlich tumor cells found in regional lymph nodes 30 days after inoculation in the footpad have a smaller nuclear volume in comparison to those that grew in the footpad. The high standard deviations of the mean in both groups reflect the great diversity of cellular phenotypes, denoted by intense cellular pleomorphism.

About the DNA quantitation, the Feulgen's method was used to evaluate indirectly the nuclear ploidy. The tumor cells harvested from the popliteal lymph node one hour after their inoculation in the footpad showed comparable DNA content to the original ascitic tumor, as it was expected. This finding asserts the authenticity of this biological assay. At

this time, the tumor cells migration occurred probably by the mechanical opening of the footpad lymphatic channels.

On day 30, the DNA content of the footpad tumor cells was greater than the original ascitic tumor cells (OT). We can propose that the development of the solid Ehrlich tumor in the footpad might genetically select the growing tumor cells. On the other hand, LN30D cells presented comparable DNA content to the F30D tumor cells, suggesting the abscence of a selective effect for this characteristic in the present model of lymphatic dissemination. The high standard deviation obtained in this group may also be related to the tumor cells pleomorphism.

It should be noticed that these results are related to both the cells that migrated to regional lymph nodes and those that determined metastatic foci.

The results of the DNA quantitation are in accordance with those obtained in rats by OLINICI et al. [11] (1977), who found the same chromosome counts in ascitic and lymph nodes Ehrlich tumor cells (major modal number of 44 chromosomes and a minor mode of 75-85 chromosomes). The analysis of banded metaphases would be of interest to compare primitive and lymph node tumor cells in this model of lymphatic dissemination performed in mice.

The interest in identifying the characteristics of metastatic cells within the primitive tumor is not new, since this kind of information could help in predicting the clinical evolution of malignant diseases. The previous data on comparisons between primary and metastatic tumor cells were performed in clinical studies and the results are somewhat conflicting. VAN DER LINDEN et al. [18] (1986) showed by morphometrical techniques that axillary lymph nodes metastatic cells presented smaller and less pleomorphic nuclei than primitive mammary adenocarcinoma cells. Other clinical studies, however, showed that the cells in the lymph nodes are similar in nuclear dimensions to those in the primary lesion, but are different as to the ploidy [18]. In a recent investigation, ARENDS et al. [1] (1987), studied the characteristics of primitive neoplasms (colorectal carcinoma) and their lymph node metastasis, showing genotypic, but not phenotypic differences between them.

The role of regional lymph nodes in metastasis is controversial. FISHER; FISHER [8] (1965), found tumor cells leaving the lymph nodes after their inoculation in the afferent lymphatic vessels, and contested their role as true barriers. It is known, however, that some metastatic tumors can colonize a wide variety of tissues, and others can selectively colonize distal organs [20]. This preference is not thoroughly understood, and it was also reported for lymphatic metastasis [3]. Lymph nodes can elaborate an immune response for tumor cells [5] but the effectiveness of this response in killing them is doubtful.

The present report showed that simple morphometric and cytogenetic methods are able to determine some characteristics of migrating tumor cells in an experimental model of lymphatic metastasis. From the foregoing statements we think that further studies combining clinical, morphological and cytogenetical data should be encouraged in order to determine the relationship between biological behaviour and cellular characteristics in neoplasia.

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RESUMO: O tumor de Ehrlich cresce na forma sólida quando inoculado no coxim plantar de camundongos, disseminando-se para o linfonodo poplíteo. Este estudo foi realizado com o intuito de caracterizar as células tumorais que cresceram no coxim plantar e as que migraram para o linfonodo poplíteo. O volume nuclear dessas células foi medido com o auxílio de ocular cariométrica. A quantificação do DNA foi feita através de citofotometria em células coradas pelo método de Feulgen. As células tumorais encontradas nos linfonodos poplíteos uma hora após a inoculação no coxim plantar não apresentaram diferenças estatísticas do tumor ascítico original e das células tumorais do coxim plantar em relação à quantidade de DNA, no mesmo tempo experimental. As células tumorais dos linfonodos poplíteos aos 30 dias após a inoculação mostraram menor volume nuclear e a mesma quantidade de DNA do que as que cresceram no coxim plantar. As células tumorais presentes no coxim plantar 30 dias após a inoculação mostraram uma quantidade maior de DNA do que as colhidas uma hora após a inoculação. Estes resultados sugerem um possível efeito seletivo nas células do tumor de Ehrlich que crescem no coxim plantar, mas não quando de sua metastatização para o linfonodo regional.

UNITERMOS: Metástase linfática; Carcinoma de Ehrlich; DNA

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TABLE 1 - Dimensions of the longest axis (LA), shortest axis (SA) and nuclear volume (NV) of the Ehrlich tumor cells that grew in the footpad and that migrated to regional lymph nodes. Results are expressed as mean ± s.d.

	LA(u)	SA(u)	NV(u^3)
Footpad	11.06 ± 1.61	7.9 ± 1.21	469.7 ± 186.3
Lymph Nodes	10.48 ± 2.2	7.26 ± 1.52	405.3 ± 231.9

TABLE 2 - DNA content of Ehrlich tumor cells in the ascitic form (OT), in the footpad 1 hour (F1H) and 30 days (F30D) after inoculation and in the popliteal lymph nodes 1 hour (LN1H) and 30 days (LN30D) after inoculation. Results are expressed as mean ± s.d.

GROUPS	DNA content (picograms)
OT	16.1 ± 6.42
F1H	18.43 ± 4.56
LN1H	21.78 ± 7.09
F30D	24.52 ± 9.39
LN30D-	24.24 ± 14.83