Release from apoptosis correlates with tumor progression in the AKR lymphoma

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Abstract

Disturbance of apoptosis is an established factor in tumorigenesis. The role of apoptosis in tumor progression is not yet clear. In the present study we compared the tendency to spontaneous apoptosis (and the proliferative capacity) of tumor cells derived from primary (PT) and metastatic tumor (MT) cells of several AKR lymphoma variants. Apoptosis-related gene expression was also compared. Our results indicate that release from apoptosis has a role in the tumor progression of this T cell lymphoma. At the cellular level, a markedly lower apoptotic tendency was observed in MT than in PT cells. The existence of macrophages only in PT also supports the presence of apoptotic cells in local but not in MTs. By contrast, proliferative capacity does not determine tumor aggressiveness in this system. At the molecular level, we found a higher staining intensity for bcl-2 in MT than in PT cells, suggesting that bcl-2 might be responsible for the reduced apoptosis in MT compared to PT cells. Evidence for p53 overexpression was found in the MT cells of one of the variants but in none of the PT. Comparison of Fas receptor, unexpectedly showed an increased expression in MT versus PT cells, possibly indicating resistance to Fas-induced apoptosis in the MT cells. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Apoptosis resistance; Tumor progression; Primary versus metastatic tumor; Apoptosis-related gene expression; Metastatic phenotype; AKR lymphoma

1. Introduction

Cell proliferative capacity served for decades as a criterion for evaluating cancer prognosis. It also constitutes the basis for attack by chemo- and radiotherapy. Disturbance in tumor cell deletion due to apoptosis has emerged during recent years as a factor in tumorigenesis [1] and possibly in tumor progression [2] and response to therapy [3].

The regulation of apoptosis is fundamental to hematopoietic homeostasis. Therefore the acquisition of lesions having an anti-apoptotic effect may constitute an important event in the genesis and possibly evolution of hematological malignancies.

The role of release from apoptosis in tumor progression is not clear. Relatively few data exist at the cellular level and data concerning determination of apoptosis-related genes at various stages of tumor progression are conflicting.

The p53 gene induces apoptotic cell death in cells which have undergone damage to DNA. p53 modification is the most common genetic alteration in human cancer [4]. The role of p53 in tumorigenesis...
is evident from the tendency of patients with the Li-Fraumeni syndrome to develop at early ages various types of neoplasia [5]. p53 deficient mice were found to develop mainly lymphomas and sarcomas [6]. p53 has, however, been reported to play a role also in tumor progression [7], whether at early or at late [8] stages. Blyth et al. have shown a synergy between c-myc transgene and p53 null genotype in the metastatic potential of thymic lymphomas.

The survival promoting gene, bcl-2, is known to play a role in lymphomagenesis in both humans [9] and mice [10]. As for its role in tumor progression, evidence for and against an enhanced malignant behavior of tumors overexpressing bcl-2, has been presented [11–14].

T cell lymphomas are among the most aggressive lymphoproliferative diseases, and very resistant to therapy. The AKR lymphoma is considered to be a good model for human T cell non Hodgkin’s lymphomas. Interestingly, virus-associated T cell leukemias/lymphomas are characterized by a poor prognosis and rapid emergence of drug resistance [15]. We have been working in our laboratory on tumor progression models of this malignancy. One model consisted in isolating and characterizing variants differing in degree of malignancy [16–20]. Another model consisted in comparing cells derived from primary tumors (PT) and metastatic tumors (MT) of the same animal which displayed a differential biological behavior [21]. We have described differences in immunophenotype [22] and in sensitivity to cytotoxic drugs [17,23], lectins [24] and immune reactions [25], between different malignancy variants.

In the present study we have undertaken to find out whether during tumor progression of AKR lymphoma a reduced tendency to programmed cell death (PCD) is observed. We have indeed recently shown in one lymphoma variant, the TAU-47, that MT cells had a much lower content in apoptotic cells than PT cells [26]. In the present study, we first extended the previous one by comparing both the tendency to spontaneous PCD and proliferative activity between PT and MT cells of several AKR lymphoma variants. We further attempted to determine the role of apoptosis-related gene expression in the tumor progression-related reduced apoptosis.

2. Materials and methods

2.1. Animals and tumors

AKR/J mice, 6–8 weeks old, were obtained from the Tel-Aviv University Animal Breeding Center. The tumor progression model used in the present study consisted in comparing PT and MT cells derived from the same animal in four AKR lymphoma variants. The PTs consisted of the local tumors formed at the s.c. site of inoculation while for MT growths we used the tumorally enlarged mesenteric lymph nodes which consisted of huge, up to 2000 mg growths. The variants used in this study were: TAU-45, TAU-47, TAU-44 and TAU-33 in order of increasing malignancy.

Tumor cell suspensions were prepared as previously described [17]. For biological behavior experiments, equal inocula of PT or MT cells were injected s.c. in the back of mice (2 x 10^5 cells/0.2 ml RPMI medium). Tumor development was evaluated by following PT and MT growth, as well as mice mortality. Each experimental group consisted of at least five mice and experiments were repeated at least four times.

2.2. DNA flow cytometry

Cells derived from PT and MT of each of the AKR lymphoma variants were incubated with propidium iodide (50 μg/ml) following the procedure of Vindelov [27]. The data were analyzed on Cell Quest Software BP, MultiCycle Phoenix Flow Systems, Phoenix, AZ, USA.

2.3. Giemsa staining

Tumor cell suspensions were washed with PBS and the pellet was spread on slides. Cells were fixed with methanol and then stained with May–Grunwald–Giemsa. The percentage of mitotic figures, apoptotic bodies as well as macrophages were counted at high power fields. A total of 300 cells was counted.

2.4. DNA fragmentation analysis by agarose gel electrophoresis

DNA from PT and MT tumors of the AKR lym-
phoma variants were analyzed by horizontal electrophoresis during 4 h on 1.5% agarose gel and visualized by UV fluorescence after staining with ethidium bromide (0.5 μg/ml).

2.5. Analysis of Bcl-2 protein expression by flow cytometry

Single cell suspensions were washed with PBS and red blood cells were removed by RBC buffer. Cells were suspended at a concentration of 2×10^7 cells/ml in SB (1% BSA (Sigma) in PBS, pH = 7.4) and saponin 0.03% (Sigma), in order to permeabilize cells [28]. The cells were incubated with hamster anti-mouse-Bcl-2 mAb (PharMingen, USA, clone 3F11) for 30 min at 4°C. After washing with SB+saponin, cells were incubated with FITC-conjugated goat anti-Armenian hamster IgG (PharMingen, Jackson ImmunoResearch Laboratories) for 30 min at 4°C. After two additional washes samples were analyzed on a FACSORT Becton Dickinson, (San Jose, CA, USA) with WINMDI Joseph Trotter Scripps data processing. Each experiment contained its own controls, consisting of cells treated with secondary antibody only.

2.6. Analysis of p53 expression by flow cytometry

Cells prepared as above were washed in PBS and resuspended in buffer containing BSA 0.2%, Sodium azide 0.002% and Triton X-100 0.05% to a concentration of 3×10^7 cells/ml. The cells were incubated with the PAb 240 or PAb 122, mouse anti-mouse p53 mAb (Chemocon, clone 240 and NeoMarker, clone 122, respectively) for 30 min at room temperature. After washing with the buffer, the cells were incubated with PE-conjugated donkey anti-mouse IgG (PharMingen, Jackson ImmunoResearch Laboratories) for 30 min at room temperature. After washing twice with the buffer, cells were analyzed by FACS as above.

As p53 positive cells, we used the p53His175 mutant - transfected H1299 p53 null human lung cancer cells [29,30] - and the SkBr3 human breast carcinoma cells (mutant 175 codon), kindly provided by Prof. Moshe Oren and Dr. Giovanni Blandino from the Department of Molecular Cell Biology, the Weizman Institute of Science, Rehovot, Israel.

Cells from the H175 cell line were cultured in RPMI containing 10% fetal calf serum (FCS), 4 mM glutamine-penicillin-streptomycin solution, 1:100 non essential amino acids and 1 mM Sodium pyruvate. Cells from the SkBr3 line were cultured in DMEM medium containing the above mentioned supplements.

The PAb 240 antibody used by us recognizes the epitope between residues 156–214 of both human and mouse p53, including therefore the amino acid...
at the 175 site (in which arginine was substituted by histidine) [31]. The PAb 122 antibody recognizes a conserved determinant between the 370–378 amino acids at the C terminal end of the p53 protein.

2.7. Analysis of Fas expression by flow cytometry

Cells prepared as above were washed in cold buffer (1% FCS in PBS) and were suspended to a concentration of $2 \times 10^7$ cells/ml. In order to reduce FcγII/III R-mediated antibody binding, the cells were incubated with 0.25 µg Fc Block (PharMingen, clone 2.4G2) per 1000 000 cells for 3 min, at 4°C. After washing, the cells were incubated with hamster anti-mouse Fas mAb (PharMingen, clone Jo2) for 30 min at 4°C in the dark. After washing with cold buffer, cells were incubated with the same secondary antibody as above and analyzed by FACS.

2.8. Statistical evaluation

Statistical evaluation was performed using Student’s t-test.

3. Results

3.1. Comparison of the biological behavior of PT and MT cells

A comparison of the in vivo biological behavior of PT versus MT cells of the TAU-45 AKR lymphoma variant is presented in Fig. 1. Twenty days after inoculating PT and MT cells subcutaneously, the weight of spleen, thymus and lungs was compared between the two groups of mice. The average weight of all three organs (as well as that of kidney and mesenteric lymph nodes, not shown) was higher in mice inoculated with the MT cells than in those injected with the PT cells. The difference was statistically significant with regard to thymus ($P < 0.0125$) and lungs ($P < 0.01$) but not for the spleen. A higher mortality rate of the MT-bearing as compared to the PT-inoculated mice was observed (not shown). The same trend was seen when comparing the PT and MT cells of the other two variants, TAU-44 and TAU-33.

Fig. 2. Comparison of DNA flow cytometry of PT and MT cells in three AKR lymphoma malignancy variants.
Fig. 3. Comparison of content in S and G2M phase cells according to DNA flow cytometry between PT and MT cells of different AKR lymphoma variants. N.Sp. = Normal Spleen. The data represent the average of 5–7 experiments for each of the variants.

Fig. 4. Comparison of apoptotic cell content between PT and MT cells in different AKR lymphoma variants - FACS analysis. (a) DNA content; (b) forward scatter and (c) double plot of DNA content versus cell size. The data indicate the percentage of cells with DNA content below that of G0/G1. They represent the average of 6–8 experiments. N.Sp. = Normal Spleen.
3.2. Comparison of DNA flow cytometry pattern in PT and MT AKR lymphoma cells

A comparison of DNA flow cytometry in PT and MT cells of the three AKR lymphoma variants in a representative experiment is presented in Fig. 2. Very marked differences were observed between PT and MT cells in all three variants, both with regard to cell cycle phase distribution and to content in low DNA containing (apoptotic) cells.

Fig. 3 presents the average of several experiments of the cell cycle phase distribution according to FACS analysis. The PT cells of all variants had a much higher fraction of cells in the S and G2M phases (constituting around 40% of the cells) than the MT cells in which they represented only 4–8%. The difference between PT and MT cells was very marked (×8.4, ×4.7 and ×8.7 in the PT versus the MT cells for the TAU-45, TAU-44 and TAU-33 variants, respectively) and highly significant for cell cycle phase distribution and to content in low DNA containing (apoptotic) cells.

Table 1
Comparison of apoptotic cell content between PT and MT cells of different AKR lymphoma variants: FACS analysis according to DNA content and forward scatter

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>DNA content</th>
<th>Forward Scatter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apoptotic cells (%) ± S.D.</td>
<td>P (PT compared to MT)</td>
</tr>
<tr>
<td>N.Sp.</td>
<td>3.9 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>TAU-45-PT</td>
<td>32.3 ± 11.4</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>TAU-45-MT</td>
<td>7.5 ± 5.2</td>
<td></td>
</tr>
<tr>
<td>TAU-44-PT</td>
<td>37.7 ± 12.3</td>
<td>P &lt; 0.0005</td>
</tr>
<tr>
<td>TAU-44-MT</td>
<td>2.4 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>TAU-33-PT</td>
<td>28.3 ± 8.3</td>
<td>P &lt; 0.0005</td>
</tr>
<tr>
<td>TAU-33-MT</td>
<td>3.1 ± 2.5</td>
<td></td>
</tr>
</tbody>
</table>

These data represent the average of 6–8 experiments.
AI = Apoptotic index, N.Sp. = Normal spleen.
the three variants \((P < 0.0005)\). The proportion of cells in the S phase was much higher than that in the G2M phase in all cell types except for the TAU-44-MT cells.

All the tumor cells except the TAU-45-MT cells (which were close to diploid) were found to be slightly hyperploid (not shown).

A comparison of content in apoptotic cells between PT and MT cells, calculated from the DNA flow cytometry data is shown in Fig. 4a and Table 1. Cells and DNA containing particles with low DNA content (sub G1) were in much higher quantities in PT than in MT cells. The difference between PT and MT cells was highly significant in the three variants, and was particularly marked in the TAU-44 variant.

A similar trend, although with less marked differences between PT and MT cells, was seen while summarizing the data of forward scatter (Fig. 4b and Table 1). The percentage of small cells (between FACS channels 0–60) was much higher in the PT than in the MT cell population.

A double plot presentation of DNA content versus cell size (according to forward scatter) is presented in Fig. 4c. Again, the fraction of apoptotic cells, represented here by the subpopulation of small low DNA containing cells, is much higher in the PT than in the MT cells, in the three AKR lymphoma variants.

3.3. Comparison of mitotic activity, apoptosis and macrophage content in PT and MT populations according to cell morphology

Staining of tumor cells by May–Grunwald–Giemsa (Fig. 5 and Table 2) showed a higher content in apoptotic bodies in the PT cells than in the MT ones. Mitotic figures were observed only among the PT cells. Macrophages were also seen only in the PT, fitting the presence of the high amount of apoptotic bodies in these growths and their paucity in the MTs.
3.4. Comparison of DNA fragmentation between PT and MT cells

DNA fragmentation, as seen in agarose gel electrophoresis, was compared between PT and MT cells of the TAU-45, TAU-44 and TAU-33 AKR lymphoma variants (Fig. 6). A ladder type pattern of DNA fragmentation was observed in the PT cells of all three variants (lanes 2, 4 and 6). As for the MT cells, some DNA fragmentation, typical for apoptotic cells, was seen only in the TAU-45 (lane 5) but none was observed in those of the TAU-33 and TAU-44 cells (lanes 1 and 3, respectively). No DNA fragmentation was seen in the normal splenocytes (lane 7).

3.5. Expression of apoptosis-related genes in PT versus MT cells

Fig. 7 presents a comparison of Bcl-2 expression as examined by FACS analysis of cells derived from PT and MT cells. As seen in this representative experiment, the expression of Bcl-2, as shown by intensity peaks, was markedly higher in the MT than in the PT cells in all three variants. The quantitative analysis of all the experiments done with regard to the bcl-2 staining intensity in the PT and MT cells is presented in Fig. 8a. Almost four fold higher fluorescence values of the bcl-2 protein content were ob-
served in the MT as compared to the PT cells. The percentage of the bcl-2 positive population was however, on the contrary, slightly reduced in the MT as compared to the PT cells (Fig. 8b).

A comparison of the p53 mutant protein in the PT and MT cells, as detected by the PAb 240 and Pab122 antibodies is presented in Fig. 9. While the positive control line, p53His175, shows a considerable level of mutant p53, the TAU-47 PT cells appear completely negative with regard to the PAb240 and the TAU-47 MT cells are slightly positive. All the other AKR lymphoma cells, whether deriving from PT or MT, were negative (not shown).

As for the PAb122, negative results were obtained with all the variants for both PT and MT cells (Fig. 9 and results which are not shown).

Table 2
Comparison of mitotic and apoptotic indices and macrophage content between primary and metastatic AKR lymphoma cells

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Mitotic index (%)</th>
<th>Apoptotic index (%)</th>
<th>Macrophages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAU-45-PT</td>
<td>0.1 ± 0.1</td>
<td>6.1 ± 0.9</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>TAU-45-MT</td>
<td>0.0</td>
<td>2.0 ± 0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>TAU-44-PT</td>
<td>0.6 ± 0.3</td>
<td>10.3 ± 3.0</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>TAU-44-MT</td>
<td>0.0</td>
<td>0.2 ± 0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>TAU-33-PT</td>
<td>0.5 ± 0.4</td>
<td>9.2 ± 4.1</td>
<td>0.0</td>
</tr>
<tr>
<td>TAU-33-MT</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Tumor cell suspensions were washed with PBS and the pellet was spread on slides. The cells were fixed with methanol and stained with May–Grunwald–Giemsa.

The data are related to 300 cells counted at microscopic magnification 400×.
The flow cytometry analysis of the Fas receptor expression (Figs. 10 and 11) shows a higher expression of the protein, as seen by both fluorescence intensity (Fig. 11a) and proportion of Fas+ cells (Fig. 11b), in MT cells in comparison with PT cells. In addition, a slight increase in Fas expression with augmenting variant malignancy in the PT as well in the MT cells can be seen. These results, although surprising, can be explained as discussed later.

4. Discussion

While cell proliferative capacity and differentiation level of tumors have been for a century the established criteria for determining disease prognosis, tendency to apoptosis only recently emerged as a possible factor in the genesis and progression of cancer. Apoptosis is known to be of importance in the normal evolution of T lymphocytes, being responsible for the deletion of inappropriate cells in the thymus [32]. T cell lymphomas might therefore constitute a good model to study the deregulation of PCD with relation to their formation and progression.

Our present results support and extend our previous data which suggested a role of release from apoptosis in tumor progression of AKR lymphoma [26]. Using various criteria (cellular morphology, DNA flow cytometry, DNA degradation assay) we consistently found, in four AKR lymphoma variants, that MT cell populations have a much lower tendency to spontaneous apoptotic cell death than PT cells (around 4% in MT cells versus ca 33% in the PT cells). The existence of macrophages among PT cells only, also supports the presence of apoptotic cells in the local tumor but not in the disseminated growths. However the presence or absence of macrophages might have other reasons as well, such as production of substances inhibitory to macrophages [33].

By contrast, cell proliferative ability clearly does not seem to determine malignant behavior in the AKR lymphoma, since MT cells exhibited, in all variants, much lower S and G2/M phase fractions (8% at most) than the PT cells (around 33%).

This apparently unexpected result suggests that high proliferative ability is not essential for the metastatic phenotype, at least not in the AKR lymphoma system. In our T cell lymphoma system it appears that release from apoptotic cell death rather than cell proliferative capacity is involved in the metastatic phenotype.

Recently a highly malignant variant of the Nb2 T cell lymphoma line was shown to display a higher resistance to apoptosis than a non-metastasizing and a low-metastasizing variant [34].

An apparently paradoxical feature was observed with regard to the PT cells of the AKR lymphoma: they display both a high proliferative capacity and a pronounced tendency to spontaneous apoptosis. The existence of such a dual state is supported by the coexistence of cell proliferation and cell death within the same areas in various tissues [35]. An unbalanced equilibrium between the two processes, rather than their absolute values, may possibly be of importance in malignancy. Interestingly, the c-myc gene induces
a state in which both cell proliferation and apoptotic death are possible, the option depending on the availability of growth factors [36].

At the molecular level, bcl-2 appears to play a role in the progression of our T cell lymphoma model. MT cells displayed a higher intensity of staining with anti bcl-2 than the PT cells (×4.3, ×3.7 and ×2.8 for the TAU-47, TAU-44 and TAU-33 variants, respectively). The proportions of cells stained are however slightly lower in the MT than in the PT cells. It is possible that the highly expressing bcl-2 cells, even if they do not constitute the majority of the MT cell population, they might determine the aggressive behavior of the tumor as a whole. Xerri et al. [37] described a higher percentage of p53+ Reed-Sternberg cells in relapsing Hodgkin’s disease as compared to non-relapsing, but this difference was even more pronounced with regard to staining intensity.

The bcl-2 molecule was usually found to block cell death, contributing thereby to tumorigenesis [10] and tumor progression [14]. Apoptotic cell death was not however always found to be related to decrease in expression of the bcl-2 gene [38]. Overexpression of bcl-2 can both promote and impair lymphoma development [39]. Evidence for and against an enhanced aggressiveness of tumors overexpressing bcl-2 has been reported [11–14].

It was suggested that since 70–90% of the malignancies developing in p53−/− mice are lymphomas, absence of p53 may be particularly devastating in the lymphoid lineage cells [40]. Deletion of the p53 gene has been shown to be involved in the development [41] and increased metastatic rates [40,41] of T cell lymphoma. We have however found only a small involvement of mutant p53 in the malignancy of AKR lymphoma.

Examination of the Fas antigen expression revealed, surprisingly, higher levels of the receptor on the MT than on the PT cells.

According to various data in literature, aberrations in the Fas-Fas ligand apoptotic cell pathway of tumor cells can result in: (1) release from apoptosis; (2) escape of the neoplastic cells from immune surveillance; (3) counterattack of the tumor cells against the immune system; (4) induction of apoptotic cell death in the normal tissue of metastatic target organs; (5) in certain conditions, ligation of the Fas receptor has been shown to cause cell proliferation.

Tumors may be resistant to Fas-dependent apoptosis due to lack of the molecule [42,43], or part of it [44], its downregulation [43], or defects in the signalling pathway downstream the receptor [45]. Sequestration of the Fas receptor in the cytoplasm, resulting in lack of translocation of the molecule to the cell membrane [46,47] can also be responsible for resistance to Fas-mediated apoptosis. Resistance to both spontaneous and immune cytotoxicity-induced apoptosis may result from such changes.

Prominent Fas antigen expression in advanced tumors was described by other authors as well in renal cell carcinoma. Horie et al. [48] even suggested Fas targeting as a therapeutic option for advanced stages of this tumor which is refractory to the usual antineoplastic treatment modalities. AIDS-related primary CNS lymphomas (AIDS-PCNSL) show high-density surface expression of Fas antigen [49]. These authors suggested that since the brain parenchyma does not express Fas receptors, local delivery of Fas-activating molecules might be effective in treatment of AIDS-PCNSL.

In addition to the apoptosis-related genes, the expression of which we have tested, other genes might be involved in the tumor progression of T cell lymphomas of mice, such as c-myc [50–52], pvt-1 [53], pim-1 [54,55] or gfi-1 [56].

Sensitivity or resistance to apoptotic cell death in cancer might have an importance for determining tumor mass, genetic instability as well as vulnerability of the tumor cells to chemotherapy and immunotherapy. Numerous studies report on the effect of chemotherapeutic agents via induction of apoptosis [3,57] and drug resistance appears to be related to escape from apoptosis [58].

We have assessed here the presence of macrophages in the PTs and their absence in the MT growths. Bcl-2 was found by Albina et al. [59] to protect P815 murine mastocytoma cells against apoptotic cell death induced by reactive nitrogen intermediate-mediated macrophage cytotoxicity. These data support a bcl-2-dependent escape of tumor cells from innate immune surveillance. A bcl-2 protection against apoptosis of the human T cell leukemia Jurkat cell line induced by CTL was also reported [60].

Deregulation of apoptosis as a mechanism of tu-
morr progression might have practical implications both as supplying new prognostic markers as well as for the design of novel therapeutic modalities.

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