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Apoptosis in human embryonal cell carcinoma: preliminary results

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Abstract Disorders in the regulation of apoptotic cell death may contribute to cancer. Furthermore, lymphocytes are supposed to play a role in counteracting tumorigenesis by inducing apoptosis in different human tumors. In this study, for the first time, tumor cell and lymphocyte apoptosis were investigated systematically in human embryonal cell carcinoma. DNA fragmentation and DNA condensation were measured simultaneously on double-fluorescence-labeled testis tumor sections using immunofluorescence microscopy. Different apoptotic indices (AIs), based either on biochemical (DNA fragmentation) or morphological criteria (DNA condensation) alone or on a combination of both, were determined in different histological regions in and around the tumor. Using morphological criteria alone, 40–75% of all apoptotic cells were not detected. Based on previous observations this finding might be related to subsets of apoptotic cells which induce the process of DNA condensation without activation of processes responsible for DNA fragmentation. Moreover, the AIs of tumor cells and lymphocytes were highest in the tumor region, compared with regions around the tumor and distant from it; these findings are discussed in the context of the Fas/FasL system.

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Introduction

Apoptosis, as an active form of cell death, plays an important role in controlling homeostasis in differentiated tissues [5] and in deleting selectively defective cells [2]. There are two main processes which typically occur concomitantly in apoptotic cells: endonuclease-induced internucleosomal DNA cleavage [15] and condensation of nuclear DNA resulting in multiple round-shaped bodies of different size localized closed to the still intact nuclear membrane [5].

Recently, DNA fragmentation and DNA condensation were shown probably to occur independently of each other in human germ tumor cells. Those cell subsets were supposed to represent an *incomplete* form of apoptosis characterized by the occurrence of either DNA fragmentation or DNA condensation only [1]. Some authors speculate that incomplete apoptosis might be responsible for the survival of defective cells and subsequent tumorigenesis [2, 4].

To test this hypothesis, different apoptotic indices were measured in metastasized and nonmetastasized human embryonal cell carcinoma. Furthermore, the spatial pattern of apoptosis was compared between the tumor region and the regions surrounding the tumor and distant from it (control), in order to recognize influences of the growing tumor on ''normal tissue''. Finally the question arose whether conclusions about the metastatic potential of human embryonal cell carcinoma could be drawn from these data.

In this tumor entity a significantly increased amount of apoptotic cells characterized either by DNA condensation, DNA fragmentation or a combination of both processes was found in the tumor region only, concerning lymphocytes as well as tumor cells. The underlying mechanism will be discussed.

Materials and methods

Tissue

Tissue samples obtained from 14 patients with embryonal cell carcinoma were examined. The cases were selected from the files of diagnosed surgical specimens at the Department of Pathology, Military Hospital, Ulm, Germany. Paraffin-embedded tissues were fixed in 4% buffered formalin and processed by standard methods. Two consecutive 5 μm cut sections were mounted per coated slide (Superfrost/Plus, Menzel Gläser, Munich, Germany). One section was processed for the immunological in-situ-end-labeling (ISEL) of DNA fragments employing the TdT assay (terminal deoxynucleotide transferase). The remaining section was stained with hematoxylin-eosin to define the tumor region and the associated area of normal tissue under the guidance of an experienced pathologist.

ISEL and DNA counterstaining

The ApopTaq Plus Kit (Oncor, Appligene, Heidelberg, Germany) was used for ISEL as recently described [1]. In brief, after deparaffination, tissue sections were incubated with 20 μg/ml of proteinase K (Boehringer Mannheim) diluted in distilled water for 8 min at 37°C, washed in distilled water (four times), incubated with TdT mix according to the manufacturer's protocol for 60 min at 37°C in a humidified chamber, washed three times in distilled water and incubated with anti-dig-fluorescein isothiocynate (FITC) for 30 min at room temperature in a humidified chamber. Either the DNA dye 4′,6-diamidino-2-phenylindole (DAPI, final concentration 1.0 μg/ml; Serva, Heidelberg, Germany) or ethidium bromide (EB, final concentration 5 μg/ml; Sigma, Deisenhofen, Germany) was added for examination of the nucleus apoptosis morphology and incubated for 5 min. Slides were washed three times with distilled water, dried at room temperature and either mounted in glycerol/paraphenylenediamine (PPD, antifading drug, final concentration 1 mM; Aldrich, Steinheim, Germany) or stored at 4°C and mounted immediately before examination. HL-60 cells (as positive control) were treated similarly, except for omitting the first treatment step with proteinase K. For negative controls, TdT was not added to the reaction mixtures. The optimization of the method (e.g., duration of proteinase K exposure) was determined in a cascade of preliminary experiments (not shown). Cells with a FITC signal in the nucleus were considered to contain fragmented DNA.

Immunfluorescence

Morphologically, cells were differentiated into (1) non-apoptotic cells, characterized by a homogeneous distribution of DNA in the normal-sized nucleus and therefore considered to represent normal cells and (2) apoptotic cells (condensed DNA), using DAPI as a DNA-specific fluorescent dye (Fig. 1; images 2, 5, 8).

The slides were scored for the two subsets described (magnification ×400) with the aid of an epifluorescence microscope (Orthoplan; Leica, Wetzlar, Germany) equipped with a filter block for DAPI excitation (excitation 270–380 nm, emission 410–580 nm). On exchanging the filter block for the filter wheel, the same cells were examined for TdT signals (excitation 450–490 nm, emission ≥520 nm, long-pass filter), enabling us to differentiate among normal cells and apoptotic cells which either bore TdT signals or not (Fig. 1; images 1, 4, 7).

To confirm our examinations performed with conventional fluorescence microscopy, we utilized confocal laser scanning microscopy (CLSM; TCS NT, Leica, Bensheim, Germany). We used the same slides, but the DNA was stained additionally with EB, since the CLSM is not equipped with a UV laser. CLSM was run in the horizontal scanning mode using two channels for simultaneous TdT and EB excitation and detection. Images consisted of 512 × 512 pixels. Series of 16 sections with an average distance of 0.5 μm were scanned per image. The underlying algorithm led to a rendered image without any kind of amplification of pixel gray values. Overlays of the rendered images allowed exact spatial localization, with the green TdT signal changing to yellow where it was superimposed over red-stained nuclear structures (Fig. 1; images 3, 6, 9).

Histological regions examined and definition of six different AIs

Histological sections located far from the tumor region (ntfar) and containing no tumor tissue served as control. The non-tumorous tissue associated with the tumor (ntass) was compared with tissue of the tumor region (tu).

Lymphocytes were differentiated from parenchymal, stromal and tumor cells on the basis of size (lymphocytes were half to one third of the size of the other cells, as quantified using CLSM) and shape (lymphocytes were oval or round, while other cells appeared polymorphic). Additional experiments performed on several sections using immunofluorescence labeling of the CD45 epitope of B and T lymphocytes (anti-human CD45 RO and CD45 RA antibody, Fa. Dianova, Hamburg, Germany) confirmed the lymphocyte measurements.

To quantify apoptosis in embryonal cell carcinoma two groups of apoptotic index (AI) comprising six different AIs have been determined:

- AIs of cells with apoptosis criteria on one detection method, *independent* of the second detection method, or showing one or both criteria, revealing the sum of all apoptotic cells: (1) $AI_{DNA\ fragmentation}$: AI based on biochemical criteria only (DNA fragmentation) (Fig. 2a); (2) $AI_{DNA\ condensation}$: AI due to morphological criteria only (DNA condensation) (Fig. 2b); (3) $AI_{all\ apoptotic\ cells}$: sum of cells which reveal one or both criteria, representing the total amount of apoptotic cells (Fig. 2c).
- AIs of cells revealing apoptosis criteria in one detection method *exclusively*, or revealing apoptosis criteria in *both* detection methods *simultaneously*. Thus complete and incomplete apoptosis could be defined (Fig. 2d): (4) $AI_{DNA\ fragmentation\ without\ DNA\ condensation}$: cells with DNA fragmentation but without DNA condensation (= incomplete apoptosis); (5) $AI_{DNA\ condensation\ without\ DNA\ fragmentation}$: cells with DNA condensation but without DNA fragmentation (= incomplete apoptosis); (6) $AI_{DNA\ fragmentation\ and\ DNA\ condensation}$: cells with DNA fragmentation and DNA condensation simultaneously (= complete apoptosis).

Statistical analysis

A total of 1296 ± 192 cells were scored per tissue, which equates to 15 microscopic fields examined. The mean and the SD as well as significance levels (*t*-test) were calculated with the aid of statistical software (Sigma Plot for Windows, 3.0; Jandel, Erkrath, Germany).

Results

Sensitivity of apoptosis detection method

To show whether both detection methods were of comparable sensitivity, percentages of morphologically and biochemically defined apoptotic cells relative to all apoptotic cells were investigated. Exclusive biochemically defined apoptosis (DNA fragmentation detected with TdT) revealed only a proportion of all apoptotic cells in the ntfar region (75.6% ± 7.5%), ntass region (58.0% ± 9.6%) and tumor region (81.2% ± 4.1%). Exclusive morphologically defined apoptosis (labeling procedure with DAPI) also revealed only a proportion of

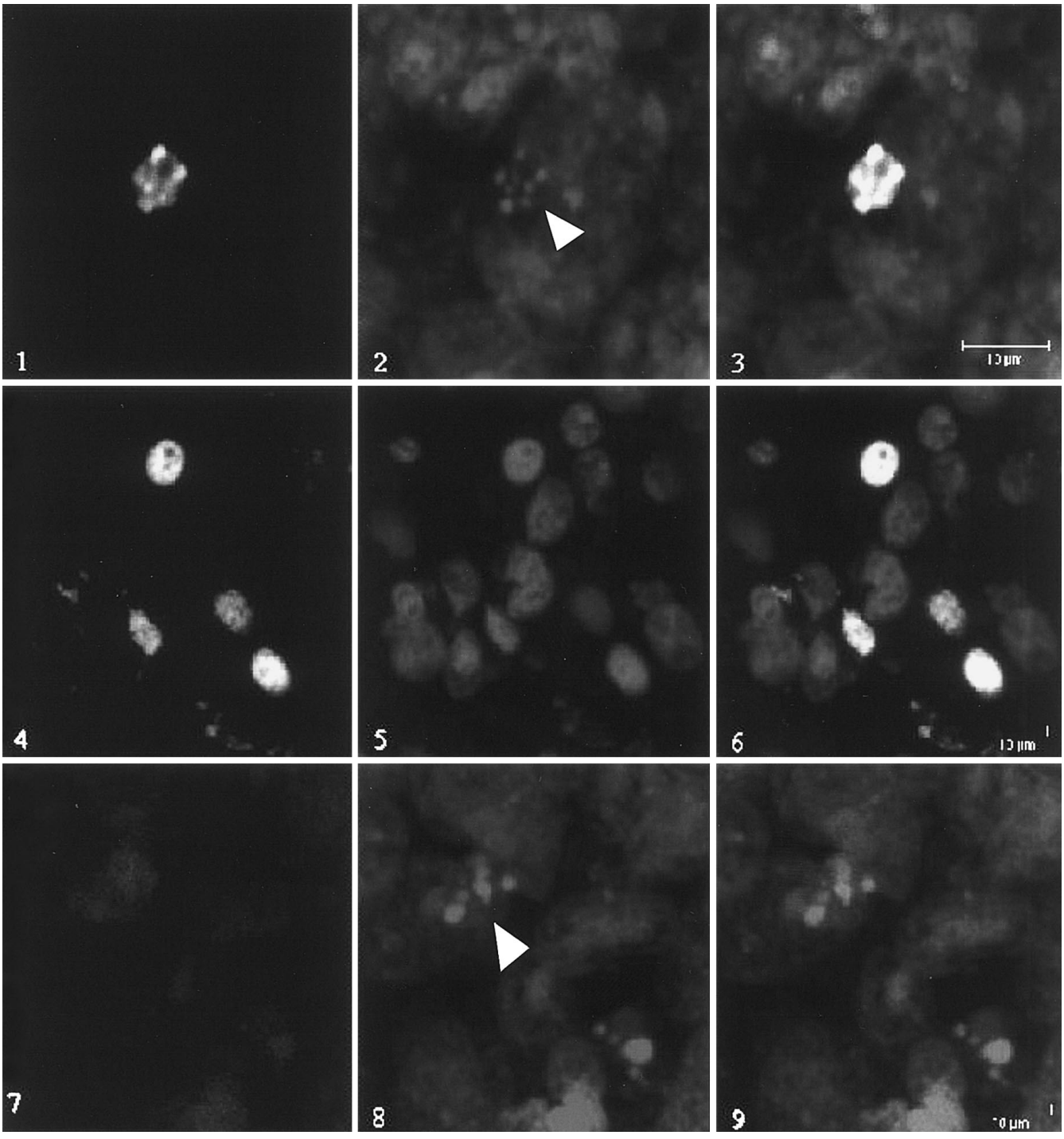

Fig. 1 Halftone photographic print of CLSM images of testis tumor cells. *Images 1*, *4*, *7* show ISEL with TdT, *images 2*, *5*, *8* show nuclear staining with EB and *images 3*, *6*, *9* represent the overlay of both methods. Testis tumor cells with DNA fragmentation (*image 1*) and DNA condensation (*image 2*, *arrow*) are shown in the first row. Second row represents cells with DNA fragmentation (*images 4, 6*), but without DNA condensation (*image 5*); third row shows cells with DNA condensation (*image 8*, *arrow*), but without DNA fragmentation (*images 7*, *9*)

all apoptotic cells in the ntfar region (25.8% ± 9.8%), ntass region (57.8% ± 9.3%) and tumor region (46.1% ± 4.5%). The amount of cells showing DNA condensation was significantly lower than that of cells with DNA fragmentation in the ntfar and tumor regions (ntfar $P = 0.002$; tumor region, $P = 0.000002$).

Cell distribution in different tissue regions

Two cell types could be identified in histological sections: lymphocytes and non-lymphocyte cells. In the ntfar and ntass tissue neither atypical testis cells nor testicular intraepithelial neoplasia (TIN) could be found

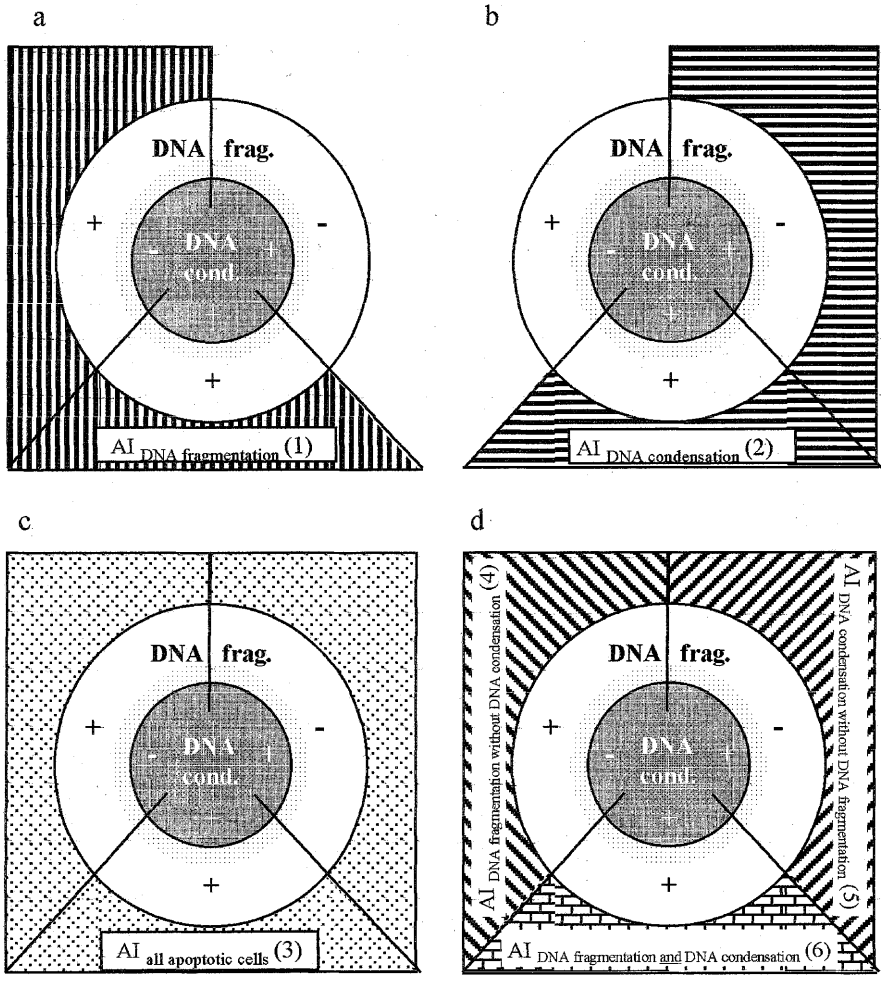

Fig. 2 Different AIs determined: **a** $AI_{DNA\ fragmentation}$: AI based on DNA fragmentation, independent of DNA condensation; **b** $AI_{DNA\ condensation}$: AI based on DNA condensation, independent of DNA fragmentation; **c** $AI_{all\ apoptotic\ cells}$: AI of cells, revealing one or both apoptosis criteria, representing all apoptotic cells; **d** *4* AI of cells with DNA fragmentation, but without DNA condensation; *5* AI of cells with DNA condensation, but without DNA fragmentation; *6* AI of cells with DNA fragmentation and DNA condensation

in HE-stained material, indicating that in these tissues the non-lymphocyte cells consist mainly of parenchymal and stromal testis cells. In the tumor region, however, nearly 100% of the non-lymphocyte cells were considered to represent testis tumor cells.

In all tissue regions examined there was nearly the same distribution of different cell types (Fig. 3). The ntfar tissue contained 18.5% ± 4.4% lymphocytes and 76.9% ± 3.9% parenchymal and stromal cells, while the ntass tissue contained 14.7% ± 10.8% lymphocytes and 81.2% ± 11.0% parenchymal and stromal cells. In the tumor tissue 16.6% ± 7.3% of all cells were lymphocytes, whereas 77.0% ± 7.1% were tumor cells.

AI dependent on histological region

The proportion of cells with *at least one sign of apoptosis*, whether DNA fragmentation or DNA condensation, was significantly higher in the tumor region than in the ntfar or ntass regions. For the *lymphocyte* fraction (Fig. 4a) the AI was significantly higher in the tumor region than in the ntfar or the ntass region regarding morphological as well as biochemical apoptosis criteria. The highest AI was measured in the tumor region for cells showing either DNA fragmentation or DNA condensation (sum of all apoptotic cells) (ntfar, 8.80% ± 6.4%; ntass, 2.82% ± 2.0%; tu, 32.73% ± 17.1%). *Non-lymphocytes* (Fig. 4b) revealed the same spatial pattern. After labeling with DAPI, however, the differences between ntfar, ntass and tumor regions were not significant.

All AIs considering *both detection methods simultaneously* were also significantly higher in the tumor region than in ntfar and ntass tissue. Focusing on the subgroup of *lymphocytes* (Fig. 5a), the differences between the tumor region and ntfar and ntass regions became significant for all AIs considering both detection methods simultaneously (for *P* values see the legend to Fig. 5a). Regarding these AIs in *tumor cells* (Fig. 5b), the same spatial pattern was shown as for lymphocytes. The difference between tumor tissue and ntfar or ntass regions, however, considering DNA condensation, was not significant (for *P* values see the legend Fig. 5b).

AI dependent on detection method

In the tumor tissue the amount of apoptotic lymphocytes and tumor cells with DNA fragmentation was

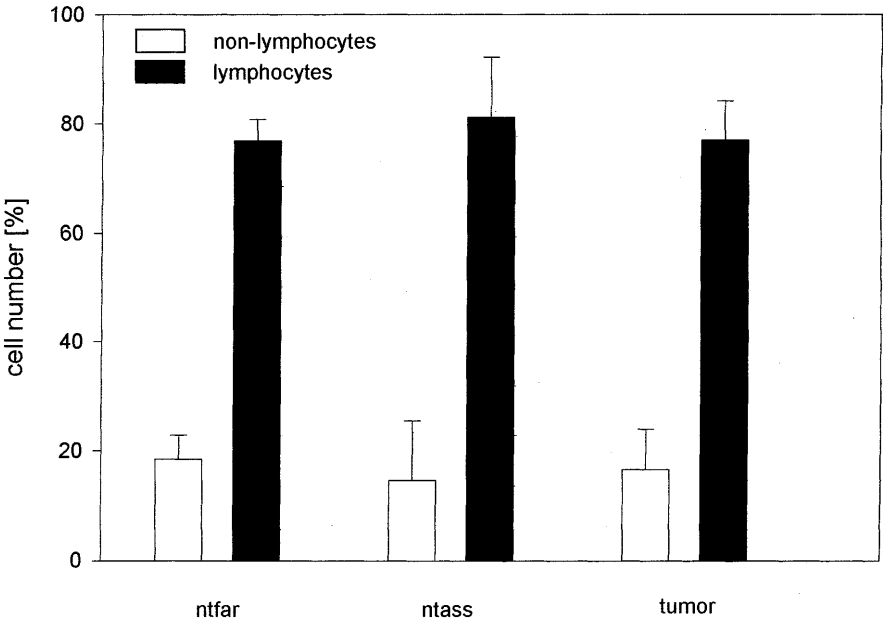

Fig. 3 Percentage of lymphocytes and non-lymphocyte cells dependent on tissue region. The sum of lymphocytes and non-lymphocytes is lower than 100% since apoptotic bodies are excluded from these measurements owing to difficulties in their assignment to lymphocytes and non-lymphocytes. (*ntfar* histological section located far from the tumor region, containing no tumor tissue, *ntass* histological section located close to the tumor tissue, but containing no tumor tissue, *tumor* histological section from tumor tissue)

significantly higher than the amount of apoptotic cells with DNA condensation. After labeling with DAPI, the AI was significantly lower than after ISEL with TdT or after a combination of both detection methods. The same was true for both lymphocytes and tumor cells (Fig. 4a, *lymphocytes*: tumor region: $AI_{DNA\ fragmentation}/AI_{DNA\ condensation}$, $P = 0.04$; $AI_{all\ apoptotic\ cells}/AI_{DNA\ condensation}$, $P = 0.003$; Fig. 4b, *tumor-cells*: tumor region: $AI_{DNA\ fragmentation}/AI_{DNA\ condensation}$, $P = 0.05$; $AI_{all\ apoptotic\ cells}/AI_{DNA\ condensation}$, $P = 0.007$). A significant difference was also found for *tumor cells* between $AI_{DNA\ fragmentation\ without\ condensation}$ and $AI_{DNA\ condensation\ without\ DNA\ fragmentation}$ ($P < 0.007$) (Fig. 5b). Concerning the *lymphocytes* no differences between the different detection methods could be found (Fig. 5a). No such difference was found in ntfar and ntass tissues.

AIs in metastasized and nonmetastasized embryonal cell carcinoma

Comparison of the AIs considering one detection method only in metastasized versus nonmetastasized embryonal cell carcinoma revealed significant differences. The AIs measured in the tumor region were always significantly higher in metastasized compared with nonmetastasized embryonal cell carcinoma (Fig. 6). No difference could be found in tissue associated with the tumor.

Discussion

Apoptosis, an active form of cell death, is an important process which plays a central role in the development of embryonal tissue and in maintaining homeostasis in differentiated tissues [5]. In the testis apoptosis occurs as an important physiological mechanism to reduce the number of germ cells in the seminiferous epithelium [6]. Disorders in the regulation of apoptotic cell death may contribute to cancer [7]. Furthermore, several studies have demonstrated the role of lymphocytes in counteracting tumorigenesis by apoptosis in different tumors such as human lung carcinomas [10], human dysgerminoma and human seminoma [1, 16]. In this study apoptotic tumor cells and lymphocytes were investigated for the first time systematically in human embryonal cell carcinoma.

Apoptotic cells are characterized by two important processes (Fig. 1): endonuclease-induced internucleosomal DNA cleavage [15] and condensation of nuclear DNA [5]. DNA cleavage can be detected by ISEL with the TdT assay [3, 11]. DNA condensation is usually examined by morphological criteria after labeling with, for example, a fluorochrome such as DAPI [8, 12]. Usually, apoptosis is determined exclusively either by DNA fragmentation or by DNA condensation. The question arose, therefore, whether both methods lead to comparable results.

Our findings clearly demonstrated that only a fraction of the total amount of apoptosis was detected when utilizing either the TdT assay only or the morphologically based assay only. About 40–75% of all apoptotic cells were not detected when using morphological criteria alone. Moreover, depending on the region examined, this percentage differed significantly, making a comparison even more suspect. It is evident, however, that the type of detection method of apoptosis is essential for the interpretation and the comparison of studies concerning apoptosis in human embryonal cell carcinoma. Which detection method might be the most suitable to answer questions of clinical significance in human germ cell tumors remains to be revealed.

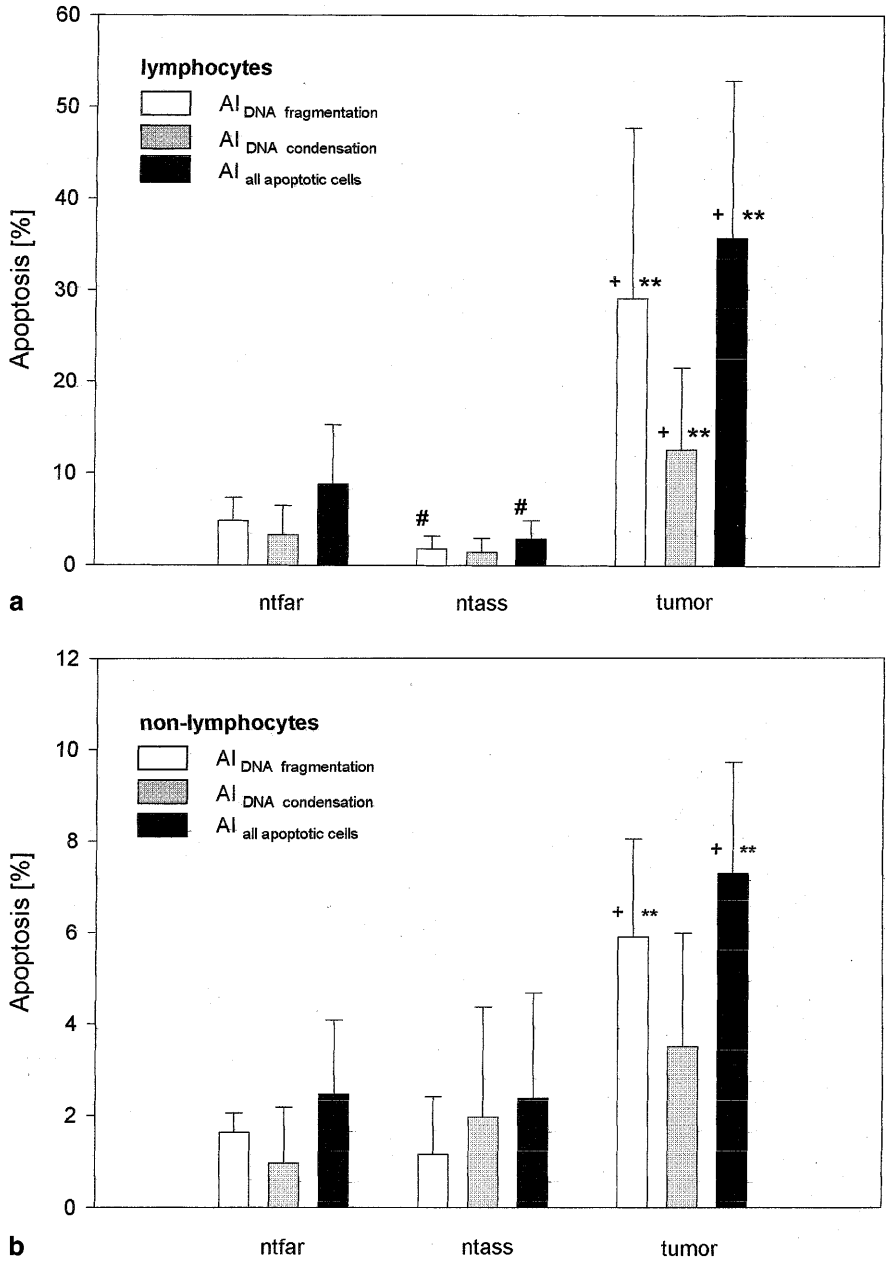

Fig. 4 **a** AI of *lymphocytes* considering one detection method dependent on histological region (# = difference ntfar/ntass: $AI_{DNA\ fragmentation}$: $P = 0.02$, $AI_{DNA\ condensation}$: n.s., $AI_{all\ apoptotic\ cells}$: $P = 0.03$; + = difference ntass/tu: $AI_{DNA\ fragmentation}$: $P = 0.001$, $AI_{DNA\ condensation}$: $P = 0.004$, $AI_{all\ apoptotic\ cells}$: $P = 0.0001$; ** = difference ntfar/tu: $AI_{DNA\ fragmentation}$: $P = 0.03$, $AI_{DNA\ condensation}$: $P = 0.05$, $AI_{all\ apoptotic\ cells}$: $P = 0.007$). (*ntfar* histological section located far from the tumor region, containing no tumor tissue, *ntass* histological section located close to the tumor tissue but containing no tumor tissue, *tumor* histological section from tumor tissue). **b** AI of *non-lymphocytes* considering one detection method dependent on histological region (+ = difference ntass/tu: $AI_{DNA\ fragmentation}$: $P = 0.00009$, $AI_{DNA\ condensation}$: n.s., $AI_{all\ apoptotic\ cells}$: $P = 0.001$; ** = difference ntfar/tu: $AI_{DNA\ fragmentation}$: $P = 0.004$, $AI_{DNA\ condensation}$: n.s., $AI_{all\ apoptotic\ cells}$: $P = 0.005$. (*ntfar* histological section located far from the tumor region, containing no tumor tissue, *ntass* histological section located close to the tumor tissue but containing no tumor tissue, *tumor* histological section from tumor tissue)

Interestingly, all AIs measured were always significantly higher in metastasized tumors compared with nonmetastasized tumors (Fig. 6). This may not serve as a prognostic parameter to predict metastasis in embryonal cell carcinoma, but it gives evidence that focusing investigation on apoptosis in testis tumors may contribute to the discovery of prognostic parameters.

An interesting feature is the significantly different AIs of tumor cells with either DNA fragmentation or DNA condensation solely in the tumor region (Fig. 5b). This contrasts with one of the classic models of apoptosis, the leukemia cell-line HL 60, where most of the tumor cells showed DNA fragmentation and DNA condensation simultaneously. As reported recently, in testis tumors genetic programs for DNA cleavage and DNA condensation might become activated independently of each other. Evidence for this was based especially on the finding that most of the apoptotic bodies representing the final stage of the apoptotic process revealed no DNA fragmentation. At least at this stage the occurrence of both apoptotic features would have been expected. Therefore it was assumed that the fraction here called "incomplete apoptotic cells" might be proportional to the fraction of tumor cells showing either DNA fragmentation or DNA condensation alone. [1]. Immunhistochemical staining always portrays a snapshot of the situation in the tumor. The findings mentioned above, however, strongly suggest that many of the tumor cells with only one apoptotic feature – DNA fragmentation or DNA-condensation – never show the second one,

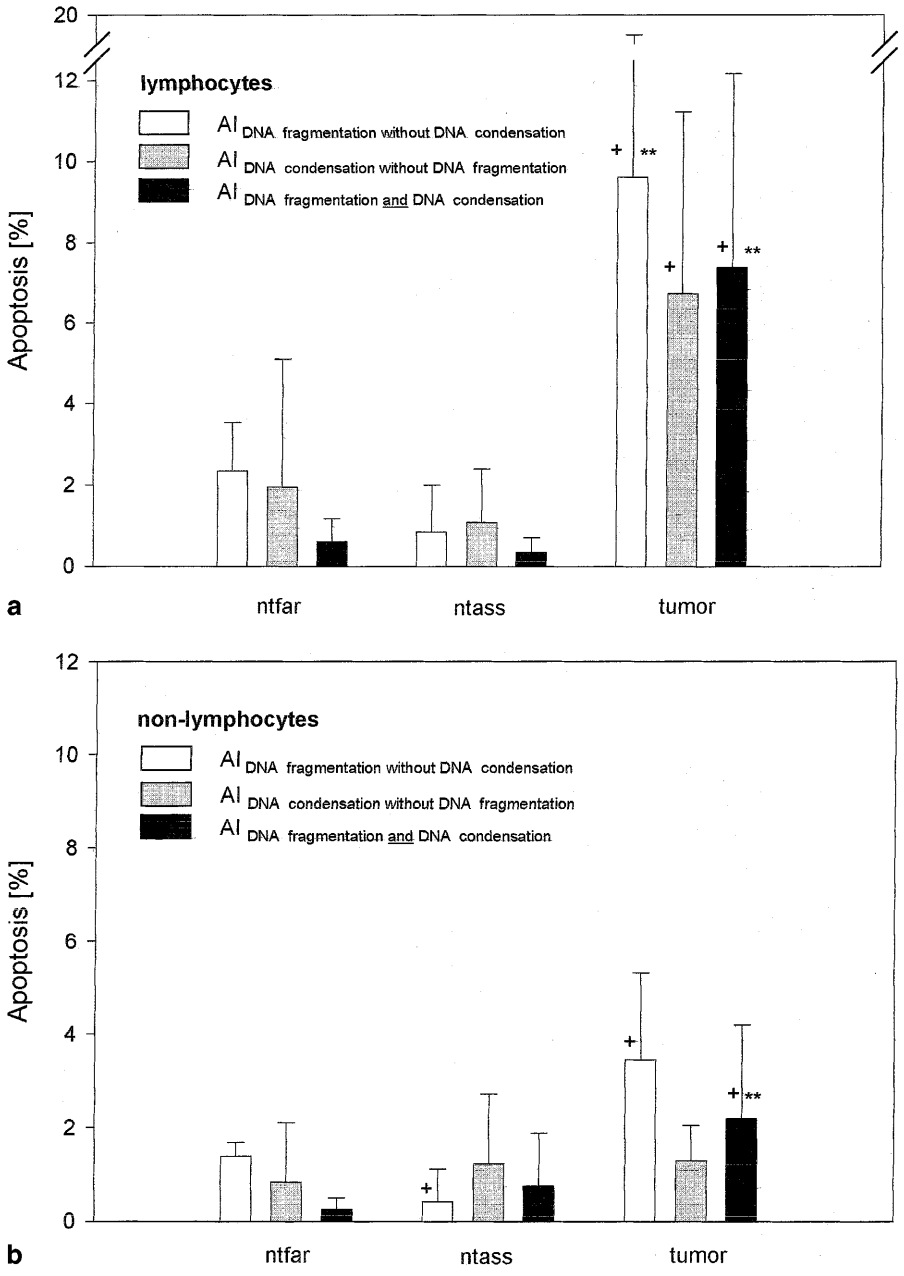

Fig. 5 **a** AI of *lymphocytes* considering both detection methods simultaneously dependent on histological region (showing incomplete and complete apoptosis). (+ = difference ntass/tu: AI_{DNA} fragmentation without DNA condensation: $P = 0.0006$, AI_{DNA} condensation without DNA fragmentation: $P = 0.005$, AI_{DNA} fragmentation and DNA condensation: $P = 0.001$; ** = difference ntfar/tu: AI_{DNA} fragmentation without DNA condensation: $P = 0.05$, AI_{DNA} condensation without DNA fragmentation: n.s., AI_{DNA} fragmentation and DNA condensation: $P = 0.02$). (*ntfar* histological section located far from the tumor region, containing no tumor tissue, *ntass* histological section located close to the tumor tissue but containing no tumor tissue, *tumor* histological section from tumor tissue). AI of *non-lymphocytes* considering both detection methods simultaneously dependent on histological region (showing incomplete and complete apoptosis). (+ = difference ntass/tu: AI_{DNA} fragmentation without DNA condensation: $P = 0.0008$, AI_{DNA} condensation without DNA fragmentation: n.s., AI_{DNA} fragmentation and DNA condensation: $P = 0.05$; ** = difference ntfar/tu: AI_{DNA} fragmentation without DNA condensation: n.s., AI_{DNA} condensation without DNA fragmentation: n.s., AI_{DNA} fragmentation and DNA condensation: $P = 0.05$). (*ntfar* histological section located far from the tumor region, containing no tumor tissue, *ntass* histological section located close to the tumor tissue but containing no tumor tissue, *tumor* histological section from tumor tissue)

remaining "incompletely" apoptotic. Several authors speculate that incomplete apoptosis might be responsible for the survival of defective cells and subsequent tumorigenesis [2, 4]. This might be true for human embryonal cell carcinoma as well.

Furthermore a specific spatial distribution of apoptotic cells in human embryonal cell carcinoma was found. Although the proportion of lymphocytes and non-lymphocytes did not differ between normal tissue (ntfar), tumor-associated tissue (ntass) and tissue of the tumor region (tu) (Fig. 3), the AIs of both lymphocytes and non-lymphocytes were significantly higher in the tumor region than in the other regions. This was independent of the type of AI (DNA fragmentation or DNA condensation) measured (Fig. 4) and it remained significant when focusing on the subfraction of cells representing incomplete and complete apoptosis (Fig. 5).

It is well known that lymphocytes counteract tumorigenesis [10, 16]. The fact that AIs of lymphocytes and tumor cells were significantly higher in the tumor region might give a clue to a lymphocyte – tumor cell interaction restricted to the tumor region. In contrast, examinations on seminoma revealed changes in the ntass region probably induced by the tumor region (unpublished data). A possible mechanism of interaction might be the Fas/Fas ligand (FasL) system. FasL, a membrane-type cytokine, induces apoptosis in target cells through its cell surface receptor, Fas. FasL is expressed in T lymphocytes, thus executing the organism's antitumor response by inducing apoptosis in tumor cells

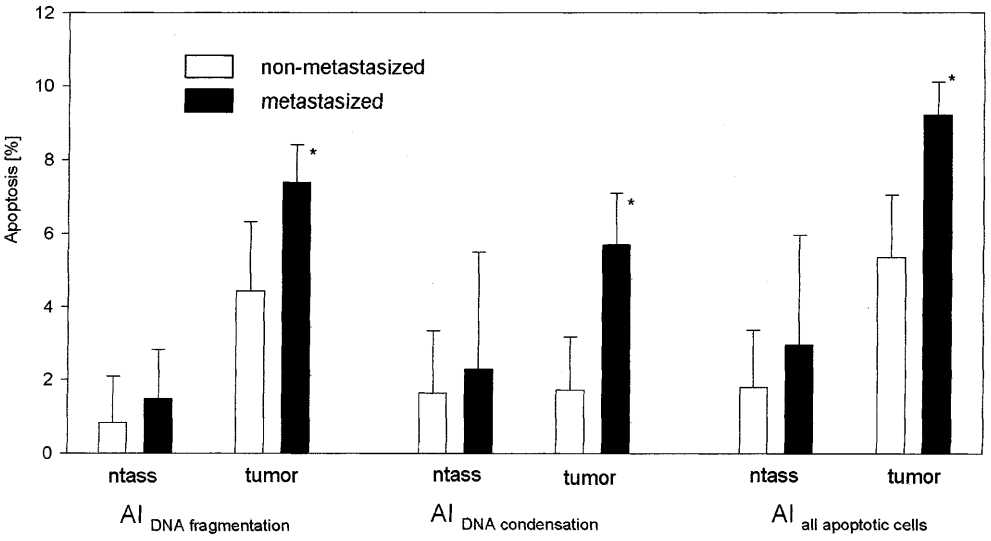

Fig. 6 AI of non-lymphocytes in metastasized and non-metastasized embryonal cell carcinoma considering one detection method. (* = difference metastasized and non-metastasized: $AI_{DNA\ fragmentation}$: $P = 0.05$, $AI_{DNA\ condensation}$: $P = 0.007$, $AI_{all\ apoptotic\ cells}$: $P = 0.005$). (*ntass* histological section located close to the tumor tissue but containing no tumor tissue, *tumor* histological section from tumor tissue)

[13]. On the other hand lymphocytes may express Fas as well, thus becoming the target of another cell bearing FasL [9]. Recently, the expression of FasL was shown in human seminoma and embryonal cell carcinoma [14]. FasL thus gives the tumor the possibility to protect itself against lymphocyte attack. Both the attack of lymphocytes against tumor cells and that of tumor cells against lymphocytes correlate well with the high AI shown in tumor regions in this study. Experiments in progress will reveal whether the Fas/FasL system is one factor responsible for this interaction.

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