FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 45, No. 2, 2007
pp. 87-91

Expression of FAS/APO 1/CD 95 in thyroid tumors

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Abstract: Using immunohistochemistry, Fas/Apo-1 protein expression was investigated in thyroid cancers of 67 patients. Thyroid biopsies from twenty eight patients with benign thyroid diseases were also examined. The patients with thyroid cancer manifested a variable histology of the cancer, including 14 patients with follicular carcinoma, 48 with papillary carcinoma, 5 patients with medullary carcinoma. The benign thyroid disease involved nodular goitre in 11 patients and follicular adenoma in other 17 patients. The study aimed at examining immunohistochemical expression of Fas protein in order to determine whether the level of its expression correlated with histological diagnosis. In individual patients Fas expression was more prevalent in thyroid carcinomas as compared to benign tumors ($p=0.001$). A marked increase in Fas expression was found in papillary carcinoma, as compared to follicular and medullary carcinomas ($p=0.02$). In conclusion, Fas was significantly more frequently overexpressed in thyroid cancer, indicating its role in thyroid tumorigenesis.

Key words: Thyroid cancer - Apoptosis - Fas protein

Introduction

Apoptosis is a basic biological process which promotes survival of the organism at the expense of individual cells and is widely used by multi-cellular organisms to remove undesirable cells [1,9,13,18,20]. Execution of the programmed death is a complex, tightly regulated, and active cellular process whereby individual cells are triggered to undergo self-destruction in a manner which will neither injure neighboring cells nor elicit any inflammatory reaction [4,7,13]. Apoptosis frequently occurs in human tumors and seems to be a significant component of the continuous cell loss which usually takes place during tumor development. Techniques for measuring apoptosis are based on morphological approach (light, fluorescence, and electron microscopy), immunohistochemistry aimed to detect apoptosis-associated proteins, and analysis of DNA degradation (terminal transferase-mediated dUPT nick-end labelling or TUNEL assay, *in situ* nick translation, and in situ hybridization for detection of DNA strand-breaks) [11].

Fas (also referred to as APO-1 or CD95) is a 48-kDa type I membrane protein, with a cysteine rich extracellular domain of 155 amino acids, which is expressed in various rapidly proliferating cells, including thymocytes, immunocompetent T lymphocytes, epithelial cells of the skin and gut [14]. Fas protein is a member of the tumor necrosis factor (TNF) superfamily and mediates apoptosis when cross-linked with agonistic anti-Fas antibody or Fas ligand (FasL) [1,13,19,24]. Fas is a widely expressed protein, found on the plasma membranes in most tissues, including thyroid. Fas-mediated apoptosis is thought to be involved in several inflammatory diseases, autoimmune diseases and cancers [5,7 8,10,12,21,22,25].

Thyroid tumors represent a good model for identification of genetic changes involved in tumorigenesis. They exhibit a stepwise progression from hyperplasia to solitary nodule, differentiated and anaplastic carcinoma.

Deregulation of the normal programmed cell death mechanism plays an important role in the pathogenesis and progression of thyroid tumors. The significance of apoptosis in thyroid carcinoma remains basically unknown. Our findings indicate that a broad spectrum of neoplastically transformed cells express Fas signal with significant variation, ranging from absent or few scarcely positive cells to the predominantly positive areas in specific histopathological types of thyroid cancers.

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Materials and methods

The study was performed on samples of thyroid lesions originating from 95 persons. The material included 28 benign lesions (11 cases of nodular goitre [NG] and 17 cases of follicular adenoma [FA]). In other 67 patients malignant tumors were diagnosed (14 cases of follicular carcinoma [FC], 48 cases of papillary carcinoma [PC] and 5 cases of medullary carcinoma [MC]). The respective paraffin blocks originated from the Department of Pathological Anatomy, University Medical School in Wroclaw and from the Department of Pathomorphology, Lower Silesia Centre of Oncology in Wroclaw. All the patients were subjected to surgery in the period of 1992-2002 due to clinical symptoms of nodular goitre in the absence of hyperthyroidism and on the basis of previous thin needle biopsy or intraoperative histopathological examination. The type of lesions was microscopically diagnosed according to the World Health Organisation (WHO) criteria [6]. Eighty six patients were males and nine were females. The median age was 63.1 years (range: 16-83 years). Before the surgery, none of the patients was subjected to preoperative radiotherapy or chemotherapy.

Surgical specimens were fixed in 5% neutralized formalin and embedded in paraffin according to a standard protocol. Serial 4 μm thick sections were cut and used for routine histological examination and for immunohistochemical analysis. For histopathological examination the sections were stained with hematoxylin and eosin. For immunohistochemic analysis the sections were mounted on sialinized glass slides, deparaffinized in xylene and washed in 100% alcohol. Endogenous peroxidase was blocked with 3% H_2O_2 for up to five minutes. The sections were washed in water and heated in mM citrate buffer (pH=6.0) for 40 min at 98°C in a water bath. The slides were cooled to room temperature, washed in Tris buffered saline (TBS), pH=7.6. The sections were incubated for 30 min with monoclonal mouse anti-human Fas/APO-1/CD95 antibody (clone: Dx2 1:500 dilution, DAKO, Denmark). After washing in TBS, pH=7.6 the signal was visualized using the catalysed signal amplification system (CSA; K 1500 KIT; DAKO, Denmark), according the procedure suggested by the manufacturers. The sections were counterstained with hematoxylin and mounted.

In every case the Fas staining was graded in areas of normal thyroid tissue, benign neoplasia and cancers. The staining was scored as membranous, cytoplasmic or mainly mixed (both patterns were observed in the same cell). At first under a light microscope, at a low magnification (×40), the studied lesions were screened to select regions which manifested the highest number of apoptotic cells (hotspots). The cells were counted using ×400 magnification. Fas-positive cells were quantified using Olympus BX 50 light microscope with visual mode and MultiScan 5.10 software for computer-assisted image analysis. In every analysed case, total numbers of thyroid cells present within the ×400 magnification field were scored. Percentage of apoptotic cells was calculated to yield an apoptotic index (*AI=number of apoptotic thyroid cancer cells/total number of thyroid cancer cells x 100*). In every case, the measurements were performed in 10 representative fields.

Results were subjected to statistical analysis using Mann-Whitney U test. For benign and malignant lesions, appropriate mean values and standard deviations were calculated for the number of apoptotic cells. Differences at the level of $p<0.05$ were regarded as statistically significant.

Results

There was no detectable Fas immunoreactivity in normal thyroid tissue adjacent to nodular lesions nor in the normal control specimens. The data strongly supported the notion that Fas expression occurred significantly more often in carcinomas (Table 1). The relationship between the frequency of apoptotic cells and the tumor histological type, classified according to WHO criteria, is displayed in Fig 1 and 2. We have demonstrated by immunohistochemistry that the expression of Fas/APO-1/CD 95 has been up regulated in PC (Fig. 6a and 6b) as compared to other thyroid carcinomas [FC, MC] (Fig. 5) and, especially, as compared to benign thyroid nodular diseases [NG, FA] (Fig. 3 and 4). Fas/APO-1/CD 95 expression has been significantly down regulated in thyroid premalignant lesions (Fig. 2). Increased number of Fas immunolabelled cells has been shown in almost all cases of papillary thyroid carcinomas (Fig. 1).

Table 1. Number of patients presenting or not Fas protein expression in nodular thyroid lesions as related to histological type of the lesion

Histological type	Nodular goiter (NG)	Follicular adenoma (FA)	Follicular carcinoma (FC)	Papillary carcinoma (PC)	Medullary carcinoma (MC)
Apoptosis Fas negative	11	6	2	0	4
Apoptosis Fas positive	0	11	12	48	1

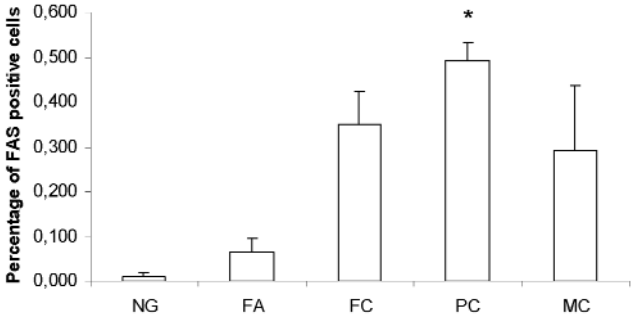


Fig. 1. Percentage of Fas protein expression in thyroid tumors: nodular gitre (NG), follicular adenoma (FA), follicular carcinoma (FC), papillary carcinoma (PC), medullary carcinoma (MC). Significant differences: PC as compared to FC and MC (*p=0.02).

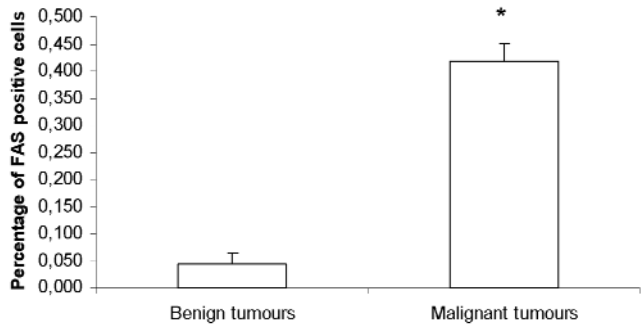
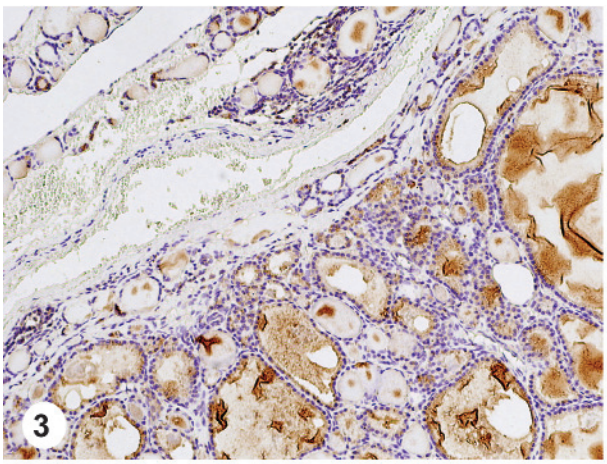
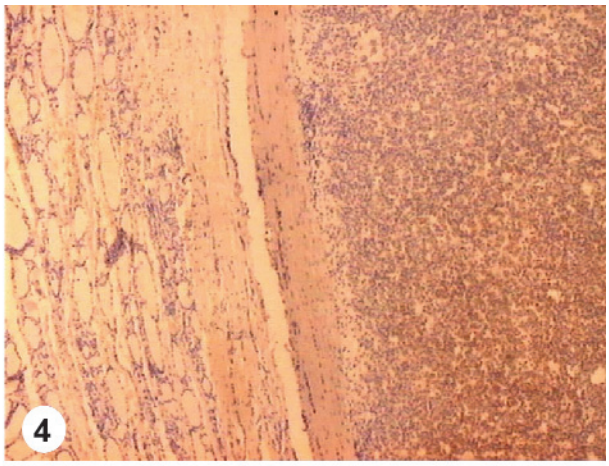
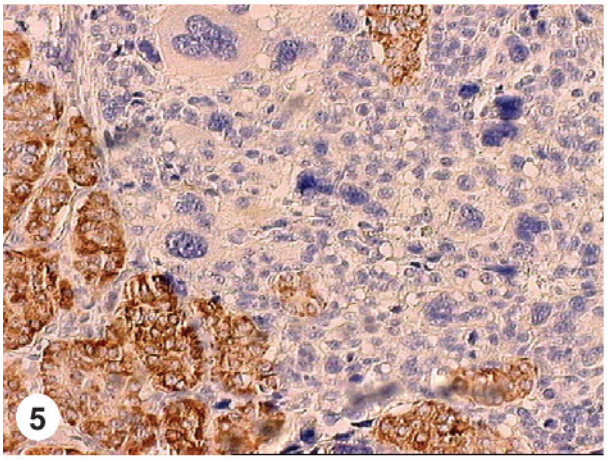
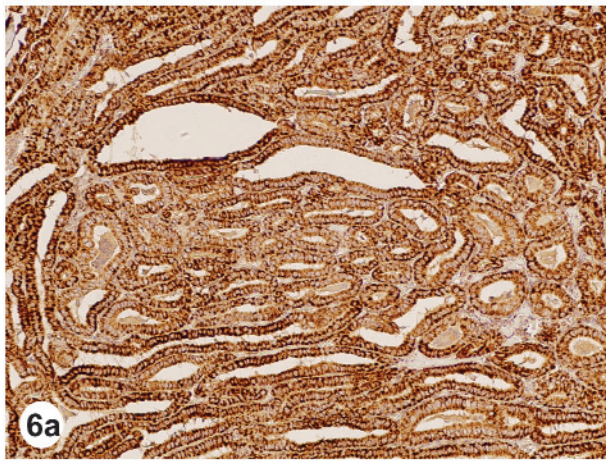


Fig. 2. Percentage of Fas protein expression in benign and malignant thyroid tumors. Significant differences: benign tumors as compared to malignant tumors (*p=0.001).









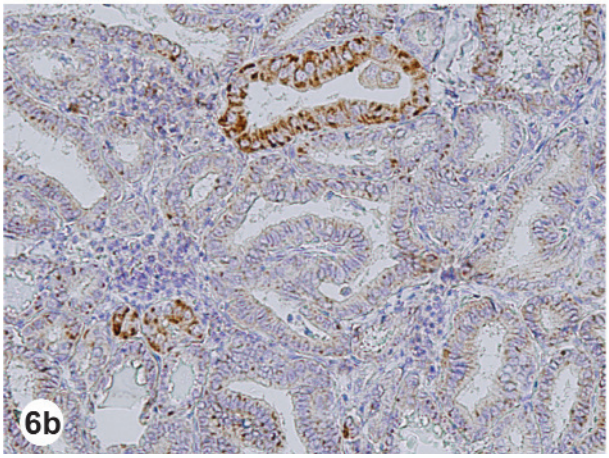


Fig 3. Large area of nodular goiter. No one cell show positive expression of CD 95 protein (magnification ×100). **Fig 4.** Complete lack of positive reaction with CD 95 antibody in follicular adenoma cells (magnification ×100). **Fig 5.** The case of insular carcinoma. In follicular areas is seen high expression of CD 95, the anaplastic pattern of carcinoma shows no reaction in contrast (magnification ×200). **Fig 6a**. Diffuse intense reaction with CD 95 antibody was seen in majority cases of papillary thyroid carcinoma. Immunohistochemistry method stain (magnification ×100). **Fig 6b**. Only focal positive reaction to CD 95 antibody in papillary thyroid carcinoma (magnification ×200).

Although no significant differences in apoptotic index (AI) could be detected between the individual thyroid histopathological processes, graphic presentation of the relationship manifested an evident disparity: the value of AI was very low in the benign thyroid lesions [NG and FA] and definitely high in thyroid carcinomas [FC, PC, MC] (Fig. 1 and 2). The difference between the benign thyroid lesions and thyroid carcinomas was significant (p=0.001). As expected, the value of AI was the highest in papillary carcinoma [PC], as compared to the other evaluated processes (p=0.02). (Fig 1).

Discussion

Apoptosis occurs in a variety of physiological situations, including embryogenesis; it plays a crucial role in normal tissue homeostasis and neoplasia [7]. A number of investigators have pointed out to the possibility that a relatively low rate of apoptotic cell death

leads to tissue-proliferative disorders, whereas a high rate of apoptosis leads to degenerative, tissue-degrading disorders [4,20]. Recent studies have analyzed tumor development in terms of disordered apoptosis in a variety of human malignancies, including thyroid cancers [1,2,3,15,26].

In this study aberrant density of Fas expression has been immunohistochemically detected and it has shown quantitatively characteristic features in different lesions. This result suggests that CD 95(Fas/Apo-1) is a biological marker, typical of papillary thyroid carcinoma and it allows to distinguish papillary carcinoma from thyroid carcinoma of different histological types. As a sensitive marker, it could reveal even scanty foci of papillary carcinoma resistant to therapy. In the present work, accumulation of Fas protein was variable, often diffuse and most commonly associated with malignant tumors of better prognosis, regardless of the adjacent non neoplastic thyroid tissue. This phenomen was confirmed in the work by Mitsiades *et al.* [16], using the immunohistochemical method of Fas labelling for the detection of apoptosis. They found that in neoplastic tissue Fas was present in 18 of 18 papillary thyroid carcinomas. But, in contrast to our results, they found Fas in 4 of 5 follicular, 1 of 1 anaplastic and 4 of 5 medullary carcinomas. They also suggested that Fas expression in almost all thyroid carcinomas of all histological subtypes represented Fas incapable of inducing apoptosis in these tumors. A similar situation was described by Arscott *et al.* [2]. They proved that papillary thyroid carcinoma demonstrated very high levels of Fas expression, as compared to the surrounding normal follicles but authors explained this just as a non functional overexpression of Fas. This up-regulated Fas was thought to be induced by lymphocytic infiltrates in thyroid papillary cancer which contained cytotoxic T lymphocytes [CTL]. The CTL were supposed to bind to Fas, initiating the apoptotic signal [23]. The authors claimed that Fas expression in thyroid cancers, including papillary thyroid carcinoma, was present and functional but overexpression of Fas was upregulated by and dependent on CTL contained in the tumor. Authors of both studies also suggested that this excessive expression of Fas may be a consequence of early events in the development of thyroid malignancies and suggested that expression of Fas protein in papillary thyroid carcinoma had an intimate relationship in particular with this histological type of tumor and especially with early events of its development, potentially playing an important role in the pathogenesis. Therefore, it was suggested that presence of Fas alone may be insufficient to induce apoptosis in cells while upregulation of Fas-induced apoptosis in thyroid may represent a partially reversible process, more complex than thought previously and, consequently, Fas-mediated stimulation does not always result in cell death [13]. Fas can also act as a costimulatory factor in the generation of an immune response [1,9,20,22].

Several experimental data were carried on cancers other than thyroid carcinomas and expression of Fas or his ligand, FasL, was noted to be decreased in correlation to the extent of malignancy in tumor cells. The data suggested that tumor cells become resistant to apoptosis during disease progression [2,16].

In the latest work Mitsiades *et al.* [15] experimentally demonstrated, why expression and activation status of Fas pathway mediator (Fas associated death domain, procaspase 8, procaspase 9 and procaspase 3) is always present and structurally intact in thyroid carcinoma cells and why it does not lead to apoptosis. They projected interesting hypothesis that carcinoma cells avoid Fas mediated apoptosis and also divert signaling pathway from cell death to proliferation.

Fas expression in thyroid cancers has been reported with quite variable frequencies [14]. Evaluation of apoptosis in thyroid tumors is carried out employing a range of techniques and the obtained results vary extensively. Different apoptotic pathways have been described and, therefore, numerous distinct techniques have been used to identify apoptotic cells [4,5,14] In assessments employing immunohistochemical evaluation of Fas expression in cancers, the obtained mean apoptotic index was significantly higher, allowing even the use of a semiquantitative score, than in studies applying TUNEL method [17]. In this study the estimates of apoptotic index did not exceed 2.5. Finally, the studies show that evaluation of the apoptotic index used to be complicated by technical and methodological problems.

Our study also implies that possibly the upregulation of Fas expression may serve as a useful biomarker in recognizing papillary thyroid cancer from other types of thyroid neoplasms. Further studies, conducted on a larger population, are needed to clarify the significance of CD 95 [Fas/Apo-1] expression in different thyroid neoplasms.

Acknowledgements: The authors thank Maciej Potyrała, MD from the Department of Pediatric Gastroenterology, Wroclaw University School of Medicine and Teresa Szkudlarek from the Department of Pathology, Wroclaw University School of Medicine for their helpful technical support.

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Received: July 5, 2006
Accepted after revision: December 1, 2006