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Aberrant expression of caspase-14 in epithelial tumors ☆

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Abstract

Cysteine-dependent aspartate-specific proteases (caspases) are the cellular executors of apoptosis. Caspase-14 is the most divergent member of the family of mammalian caspases and displays a variety of unique characteristics. It is expressed in a limited number of tissues and has the shortest amino acid sequence within the caspase protein family. During induction of apoptosis, it is not processed, whereas terminal differentiation in skin leads to cleavage of caspase-14. Here we show that 40% of lung squamous cell carcinomas, 22% of breast cancers, and about 80% of cervical carcinomas express casapse-14. Immunohistochemistry reveals that caspase-14 is localized in areas of ongoing differentiation close to necrotic sites but is not strictly associated with the differentiation markers keratin-1/-10. Caspase-14 is neither mutated nor alternatively spliced in the tumors analyzed. Furthermore, caspase-14 is not processed into a small and large subunit, a process critical for the proteolytic activation of known effector caspases. We conclude that conditions exist in tumors leading to re-expression of this normally silent gene.

Keywords: Cancer; Apoptosis; Cell death; Differentiation; Tissue necrosis

Apoptosis, or programmed cell death, is crucial for the development and homeostasis of multicellular organisms, whereas an imbalance in apoptosis can lead to diseases such as autoimmunity and cancer [1]. A key component of this regulatory network is a proteolytic system involving a family of proteases called caspases (cysteine-dependent aspartate-specific proteases), which are the cellular executors of apoptosis [2].

In humans 11, functional caspases (caspases-1 to 10 and caspase-14) have been described [3,4]. Within the network of apoptosis, several anti-apoptotic truncated caspase-like factors also exist, including caspase pro-domains such as Pseudo-ICE, ICEBERG [5] CARD-only protein (COP) [6], human caspase-12 [7], and the catalytically deficient homologue of caspase-8 FLICE-inhibitory protein (FLIP) [8]. Another class of anti-apoptotic factors are serine protease inhibitors (serpins), which inhibit both serine and cysteine proteases by forming a covalent serpin–protease complex [9]. In various epithelial tumors, serpins such as the squamous cell carcinoma antigens 1 and 2 (SCCA 1/2), hurpin or maspin were shown to be amplified [10].

Caspase-14 is the shortest and most divergent family member of human caspases displaying 24% amino acid identity to its closest functional relative (caspase-2) [4], but it is highly conserved between humans and mice (73% amino acid identity). In addition to its short amino

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acid sequence and its low sequence identity to other caspases, caspase-14 has a number of unique characteristics. Caspases-14 has a strong but restricted expression, limited to supra-basal keratinocytes of the skin and to a few specialized cells of other organs, such as epithelial cells of the choroid plexus, the pigmented layer of the retina, and Hassall's bodies in the thymus [11]. In the skin, caspase-14 expression is tightly associated with keratinocyte terminal differentiation [12]. Furthermore, it has been demonstrated that caspase-14 is not involved in either the extrinsic or the intrinsic pathway of apoptosis, as measured by the absence of any cleavage product after induction of cell death [13,14]. Although caspase-14 can associate with certain large pro-domain caspases including caspase-8 [13], it does not act as an inhibitor of caspase-8-triggered cell death [4]. An involvement of caspase-14 in another still yet undefined alternative signaling pathway was initially proposed [13] and is consistent with the recent identification of an atypical cleavage site in caspase-14 [15]. Members of the caspase family are present as inactive pro-enzymes, which are activated by cleavage at aspartate residues in the linker region to generate active p20/p10 heterotetramers [16]. In contrast to other caspases, in vivo caspase-14 is not processed after an Asp in the linker region, but between Ile^{152} and Lys^{153} [15].

The cellular function of caspase-14 is unknown. Although caspase-14 expression is tightly restricted to a few cell types in mammals, caspase-14 was recently detected in several tumor cell lines [17]. As tumor cell lines adapt to artificial culture conditions, we investigated whether caspase-14 expression occurs in primary human tumors as well.

Materials and methods

Tumor samples. Surgically removed lung and cervical carcinoma samples were derived from patients of the General Hospital of Vienna, Department of Pathology [18]. The excised tumor material was fixed in 4% formalin for histological preparations or snap-frozen and stored in liquid nitrogen for preparation of RNA. To confirm integrity of the tumor samples, two representative sections were stained with hematoxylin/eosin, during the preparation of RNA. Tumor samples containing more than 30% of residual normal tissue or of diffuse histological types or large necrotic tumors were excluded.

cDNA synthesis, polymerase chain reaction (PCR), and real-time PCR. RNA of a panel of normal tissues 0.5 μg poly(A)$^+$ each (Clontech, USA) and 1.5 μg oligo(dT)$_{15}$ primer (0.5 μg/μl) were denatured for 15 min at 70 °C in 11 μl diethyl pyrocarbonate-treated water (AD_{DEPC}). For cDNA synthesis, the following reagents were added: 5 μl of 5× AMV buffer (Roche, Switzerland), 2.5 μl dNTP mix 10 mM each (TaKaRa, Japan), 1.5 μl RNasin (40 U/μl) (Roche, Switzerland), 1.5 μl AMV reverse transcriptase (10 U/μl) (Roche, Switzerland), and 2 μl AD_{DEPC}, and mRNA was reverse transcribed at 42 °C for 1 h. The reaction was stopped by addition of 1 μl of 0.5 M ethylenediaminetetraacetic acid (EDTA) and incubation at 95 °C for 3 min.

Primers for β2 microglobulin (β2M) were chosen as described β2M(f): 5′-GATGAGTATGCCTGCCGTGTG-3′ and β2M(r): 5′-CA ATCCAAATGCGGCATCT-3′ [19], Casp14f (5′-tgcacgtttattccacgg ta-3′) and Casp14r (5′-tgctttggatttcagggttc-3′). Quantitative PCR was performed according to LightCyclerStart DNA Master SYBR Green I (Roche), in a modified version: 3 μl cDNA was amplified in a reaction mix containing 1.5 μl DNA Master Mix, 1.8 μl $MgCl_2$ (25 mM), 8.7 μl H_2O, and 0.5 μl of each primer 10 μM in an LightCycler II (Roche) (10 min at 95 °C, followed by 55 cycles of 5 s at 95 °C, 15 s at 65 °C, and 15 s at 72 °C). The PCR efficiencies for reference and target molecules were calculated using the Relative Quantification Software Version 1.0 (Roche).

Standard PCR was performed in a total volume of 50 μl containing 5 μl of 10× PCR-buffer (ABgene, UK), 4 μl dNTP mix 2.5 mM each (TaKaRa, Japan), 2.5 μl primers 5 μM each, 1 μl cDNA (diluted 1:50 in aqua dest.), and 0.2 μl Red Hot DNA Polymerase (ABgene, UK). Amplification was performed in a thermocycler (Gene Amp PCR System 9700, Applied Biosystems, USA): 94 °C × 3 min, 35 × (94 °C × 30 s, 55 °C × 30 s, and 72 °C × 30 s), 72 °C × 7 min. For PCR amplification of overlapping fragments of the human caspase-14, the primer pairs hC14F1 (5′-CTCAGTATTGGCAACTAGG AGA-3′), hC14R1 (5′-TACCGTGGAATAAACGTGCAA-3′) and hC14F2 (5′-GCCACCATGGTAGGTGGAGATGAGATTGTG-3′), hC14R2 (5′-TAGAGAGGCCATGAGCTAGGA-3′) were used.

For sequencing, PCR products were cloned into pCR2.1-TOPO vector (Invitrogen, Netherlands).

Immunohistochemistry and immunofluorescence. Five micrometer paraffin sections were mounted on positive charge slides deparaffinized and rehydrated through a graded ethanol series. For antigen retrieval, sections were placed in 0.01 M citrate buffer (pH 6.0) and heated for 2 × 5 min at 500 W in a microwave oven. They were subsequently incubated for 15 min in 0.3% H_2O_2 to block non-specific endogenous peroxidase and then incubated in 10% sheep or goat serum in PBS/2% BSA to block non-specific binding of the secondary antibody. The primary antibody was applied at 1:5000 dilution in PBS/2% BSA anti-murine caspase-14 rabbit serum [11] or anti-human caspase-14 mouse monoclonal antibody (Upstate Biotech, USA) at 1:2000 dilution overnight. Antibody pre-incubated with recombinant human caspase-14 served as a negative control. For immunohistochemistry, sections were incubated with a secondary biotinylated anti-rabbit 1:100 (Vector, UK) or anti-mouse 1:200 (Amersham) antibodies and streptavidin–biotin–peroxidase (DAKO, USA). Antibody localization was detected with 3-amino-9-ethylcarbazole (AEC, DAKO) as a chromogen substrate. Finally, sections were washed in distilled water and weakly counterstained with Harryie's modified hematoxylin.

For immunofluorescence sections were treated as described above without endogenous peroxidase block. Anti-cytokeratin 1/10 mouse monoclonal antibody KS 8.60 (Abcam, UK) was diluted 1:100.

Secondary antibodies were applied at 1:500 dilution (Alexa 488 or 546, Alexa) for 1 h in PBS/2% BSA. Nuclei were counterstained with Hoechst (1:5000) for 5 min in PBS and finally washed in distilled water.

Western blot analysis. Tissue sections were lysed in urea lysis buffer (62.5 mM Tris/HCl, pH 6.8, 6 M urea, 2% SDS, and 0.00125% bromophenol blue), sonicated, and boiled in the presence of 5% β-mercaptoethanol. Twenty micrograms of protein lysate was separated on an 8–18% gradient SDS–polyacrylamide gel and blotted on a nitrocellulose membrane (Bio-Rad, USA). Transfer was controlled by Ponceau staining. Blots were blocked for 1 h in blocking buffer (7.5% nonfat dried milk, 2% BSA, 0.1% Tween in PBS) and incubated with anti-murine caspase-14 rabbit serum (1:2000) [11], anti-human caspase-14 mouse monoclonal antibody (Upstate biotech, USA) (1:1000), and anti-actin mouse monoclonal antibody (ICN, Schweden) (1:10,000) in blocking buffer over night. Blots were washed twice in 5% non-fat drieds milk, 0.1% Tween in PBS and incubated for 1 h with goat anti-rabbit-HRP (Bio-Rad, USA) or anti-mouse-HRP (Amersham Pharmacia, UK), followed by washing several times with PBS–Tween, then PBS, and developed with ECL chemoluminescence detection system (Amersham Pharmacia, UK).