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Original article

Ellagic acid induces a dose- and time-dependent depolarization of mitochondria and activation of caspase-9 and -3 in human neuroblastoma cells

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Abbreviations:
FACS, fluorescence-activated cell sorting
LDH, lactate dehydrogenase
Rho 123, Rhodamine 123
SEM, standard error of mean
TBS, Tris-buffered saline
XTT, (sodium 3′[1-phenylaminocarbonyl)-3,4-tetrazolium]-bis-(methoxy-6-nitro) benzene sulfonic acid hydrate
$\Delta\Psi_m$, mitochondrial membrane potential

ABSTRACT

The polyphenol ellagic acid is found in many natural food sources and has been proposed as a candidate compound for clinical applications due to its anti-oxidative capacity and as a potential anti-tumorigenic compound. The objective of the present study was to evaluate the sensitivity to and possible apoptosis mechanism induced by ellagic acid in neuronal tumor cells. As a model the human neuroblastoma SH-SY5Y cell line was used. The methods applied were bright field and phase contrast microscopy, XTT- and LDH-assays, western blot, and flow cytometric analysis of DNA degradation and mitochondrial membrane potential. Ellagic acid treatment was found to induce a reduction in cell number preceded by alterations of the mitochondrial membrane potential and activation of caspase-9 and -3, DNA-fragmentation and cell death by apoptosis. The apoptotic cell death studied was not due to anoikis since it was significant in the adherent fraction of the cells. We conclude that ellagic acid induces dose- and time-dependent apoptosis, at least partly by the mitochondrial pathway, in an embryonal neuronal tumor cell system. This finding is in agreement with previously reported data on adult carcinoma cells thus suggesting a more general effect of ellagic acid on tumor cells.

1. Introduction

Ellagic acid is a polyphenol derived from hydrolysable ellagitannins present in large amounts in many plant foods such as fruits, nuts and berries. A growing number of studies have focused on the ability of ellagic acid and related polyphenols to attenuate growth and survival of cancer cells [1–3] as well as having anti-inflammatory properties [4].

Two general apoptotic pathways have been described, the intrinsic, mitochondrial pathway and the extrinsic, death-receptor-induced pathway. Both these pathways are associated with the activation of a group of cysteine-rich proteases, so-called caspases [5]. The caspases can be divided into initiator caspases (caspase-2, -4, -8, -9 and -12), effector caspases (caspase-3, -6 and -7) and inflammatory caspases (caspase-1, -5 and -11) [6]. Activation of the mitochondrial pathway is associated with alterations in expression of pro- and anti-apoptotic members of the Bcl-2 family, decrease in mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial membrane permeabilization and subsequent release of cytochrome c and other pro-apoptotic molecules and formation of the apoptosome complex (APAF) activating caspase-9 [7]. The extrinsic pathway is associated with ligand-dependent activation of death-receptors, leading to caspase-8 cleavage and activation. Both caspase-8 and caspase-9 are initiator caspases that can activate the effector caspase-3 thereby inducing the downstream events of apoptosis such as chromatin degradation.

In previously published reports on ellagic acid and effects in tumor cells, researchers have mainly used carcinoma-derived cells, for example colon [8,9], prostate [8], [10], oral [8], cervix [11], pancreas cells [12] and the leukemic cell line HL-60 [13]. Tumorigenesis of carcinomas as well as other adult cancers frequently involves loss of function in tumor suppressor genes

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such as p53 and PTEN and activation of proto-oncogenes such as K-Ras and downstream effectors in the Ras-signaling pathway. Amplification of MYC, and deregulated expression of proteins controlling proliferation and apoptosis such as Bcl-2 are also common genetic alterations in carcinoma cells.

To address the sensitivity and mechanism behind a pro-apoptotic effect of ellagic acid in tumors with a different genetic background and of neuronal origin we have in this paper investigated the effects of ellagic acid in an embryonal tumor-derived neuroblastoma cell line. The neuroblastoma-derived cell line SH-SY5Y [14] shows no known genetic alterations in classical oncogenes, except for a recently describe mutation in the ALK (anaplastic lymphoma kinase) gene encoding a receptor tyrosine kinase [15,16]. SH-SY5Y cells exhibits normal expression of functional p53 [17], and Bcl-2 [18] and no described *RAS* gene-mutations [19]. Also, SH-SY5Y cells are near-diploid [14] and can be *in vitro* differentiated and growth inhibited by several different treatment protocols [20–23]. Thus, despite its background derived from a highly malignant tumor [14], SH-SY5Y has a number of features distinctly different from the cancer cells previously studied for ellagic acid effects. In a previous pilot study, we reported that 30 μM ellagic acid induced apoptosis in SH-SY5Y judged as TUNEL-positive staining but no mechanistic or more detailed data were presented [24]. Therefore we found it interesting to identify a mechanism for induction of apoptosis and dose-sensitivity to ellagic acid.

Our here presented data showed that ellagic acid induced a dose- and time-dependent apoptosis in SH-SY5Y, mediated by alterations in the mitochondrial membrane potential and activation of caspase-9 and -3. Furthermore, the apoptosis was initiated in adherent cells thus not by anoikis secondary to induced cell detachment [25]. However, the available data also suggests additive effects involving the extrinsic pathway or caspase-independent apoptosis pathways.

2. Materials and methods

2.1. Chemicals and reagents

Ellagic acid and staurosporine were purchased from Sigma Aldrich, St Louis, MO. Ellagic acid was diluted in 0.1 M NaOH and sterile filtered through a 0.2 μm nylon filter (SMI LabHut Ltd, Gloucester, UK). The caspase-9 inhibitor, z-LEHD-fmk and the negative control peptide, z-FA-fmk were purchased from BD Biosciences, Becton Dickinson, (Franklin Lake, NJ). Inhibitors were dissolved in DMSO (<0.2% v/v final concentration).

2.2. Cell Culture

SH-SY5Y human neuroblastoma cells [14] (ATCC CRL-2266) were grown in Eagle's minimal essential medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) of heat-inactivated foetal bovine serum (Sigma Aldrich), 2 mM L-glutamine (Gibco/Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco/Invitrogen) and cultured as previously described [24]. Cells were allowed to attach for 24 hours prior to additions of ellagic acid.

2.3. Morphologic evaluation of apoptosis

Morphological signs of apoptosis were evaluated using bright field and phase contrast microscopy. A number of 1×10^5 cells were seeded in 35 mm plastic dishes and treated as indicated in Fig. 1. The cells were examined in a Leica DMI 6000 B inverted microscope (Leica Microsystems, Wetzlar, Germany) and photographed with a digital camera. Apoptotic cells were determined by rounded morphology, plasma membrane blebbing, formation of apoptotic bodies, pychnotic irregular cell morphology and subsequent detachment and fragmentation of cells (see Table 1). The exact time point when the visual morphologic changes were detectable varied slightly between different cell batches. Hence, cells were harvested for experiments at a certain morphologically defined degree of apoptosis (as seen in Fig. 1B), rather than at a certain fixed time point. It should be noted that in each individual experiment the sequence of events the cells underwent was reproducible, only the time varied slightly between experiments. All the time points for data based on mean values from several experiments therefore are presented as t (h) ± 2 h.

2.4. XTT analysis and LDH-release

XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(methoxy-6-nitro)benzene sulfonic acid hydrate)- and LDH (lactate dehydrogenase)-assays (Roche Diagnostics, Mannheim, Germany) were performed according to the manufacturers protocols. A number of 5×10^3 cells per well in 100 μL medium

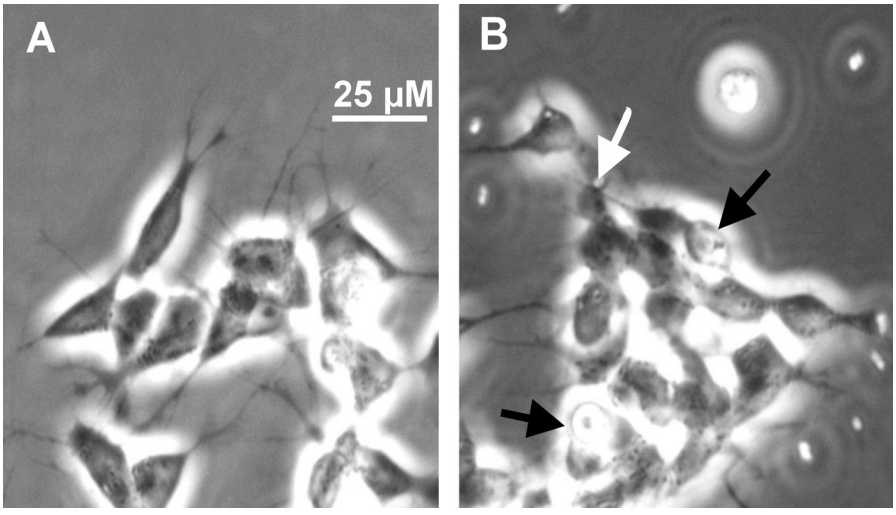

Fig. 1. Effect of ellagic acid on morphological signs of apoptosis. Cells were incubated with (A) vehicle or (B) 50 μM ellagic acid for 24 ± 2 h. The cells were examined in a Leica DMI 6000 B (Leica Microsystems) inverted microscope and photographed. Rounded up cells are exemplified by the white arrow and plasma membrane blebbing is exemplified by black arrows. Data are representative pictures from one of three experiments with duplicate samples.