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The histone deacetylase inhibitor suberoylanilide hydroxamic acid sensitises human hepatocellular carcinoma cells to TRAIL-induced apoptosis by TRAIL-DISC activation

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ABSTRACT

This paper shows that the histone deacetylase inhibitor SAHA sensitised at sub-toxic doses human hepatocellular carcinoma cells (HepG2, Hep3B and SK-Hep1) to TRAIL-induced apoptosis, while it was ineffective in primary human hepatocytes (PHHs).

In particular in HCC cells SAHA increased the expression of death receptor 5 (DR5) and caused a decrement of c-Flip. These two modifications provoked in the presence of TRAIL the rapid production of TRAIL-DISC and the activation of caspase-8. Consequently SAHA/TRAIL combination induced many apoptotic events, such as a cleavage of Bid into tBid, dissipation of mitochondrial membrane potential, activation of caspase-3 with the consequent cleavage of both NF-kB and Akt. The decrease in NF-kB level seemed to be responsible for the reduction in the content of IAP family antiapoptotic proteins while the decrease in Akt level caused a reduction in phospho-Bad. These events led to the activation of caspase-9, which contributed to the strong apoptotic activity of TRAIL.

Sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by SAHA may suggest new strategies for the treatment of liver tumours.

1. Introduction

Recently the interest of the researchers has been focused on TRAIL (Apo2L), a member of the TNF superfamily,[1] because this cytokine induces apoptosis in a number of tumour cell lines but is ineffective in the majority of normal cells.[2]

TRAIL can bind to five different receptors: four membrane-bound and one soluble receptor.[2,3] Two of these membrane receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), act as agonistic receptors, containing a cytoplasmic death domain (DD) through which TRAIL can transmit an apoptotic signal. Two other membrane receptors, decoy receptor 1 (DcR1/TRAIL-R3) and decoy receptor 2 (DcR2/TRAIL-R4), can also bind TRAIL, but act as antagonistic receptors, lacking an intact death domain. Finally the soluble receptor osteoprotegerin (OPG) binds TRAIL with low affinity.[3]

Binding of TRAIL to DR4 or DR5 results in trimerization of the receptors with the production of the death-inducing signalling complex (DISC). Through their intracellular death domains these receptors bind the adaptor protein FADD, which in turn recruits the initiator procaspase-8 to the DISC.[4] Within this complex, procaspase-8 is activated by autoproteolytic cleavage[5] with the consequent activation of effector caspases, such as caspase-3,[6] or cleavage of Bid and induction of the intrinsic apoptotic pathway.[6] Besides caspase-8, also caspase-10 can be recruited to the DISC,[7] although caspase-10

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seems to be activated in TRAIL-induced apoptosis, only when low doses of TRAIL are available.[8] Activation at the DISC of both caspases-8 and -10 can be inhibited by the cellular c-Flip, which is recruited to the DISC through FADD, thereby preventing the recruitment of caspases into the complex.[9]

NF-kB is a nuclear transcription factor which exerts a role in the control of apoptosis. This factor is assembled through the dimerization of two components selected of five different subunits: cRel, RelA (p65), RelB, p50 and p52.[10] NF-kB is predominantly found in the cytoplasm complexed with IkB-α protein. Degradation of IkB-α in response to stimuli is an obligatory step in NF-kB activation.[11] This degradation is mediated by the proteasome and requires phosphorylation of IkB-α at specific serine residues by IKK complex.[11] The released NF-kB translocates to the nucleus where it regulates the transcription of several genes involved in immune and inflammatory responses and in the control of cell proliferation, apoptosis, metastasis and angiogenesis.[12]

TRAIL has been shown, such as TNF-α, to be involved in the activation of NF-kB.[13,14] In particular TRAIL-DISC can recruit RIP-1, which causes activation of NIK and phosphorylation of IKK complex.[11] The consequent phosphorylation and cleavage of IkB-α permits the translocation of NF-kB to the nucleus, where in particular stimulates the expression of genes coding for IAP family of proteins.[15] It is noteworthy that c-Flip exerts an important role in defining whether TRAIL-DISC induces the activation of caspase-8 and apoptosis or activation of NF-kB. In fact c-Flip is not only an inhibitor of caspase-8[9] but it can recruit the proteins involved in the activation of NF-kB, favouring this process and consequently the survival of cells.[16]

It is interesting to note that the cells exhibit a different sensitivity to TRAIL. The scarce susceptibility of normal cells to TRAIL can be considered as a consequence of multiple conditions, including overexpression of the decoy receptors, low expression of the death receptors and also high levels of c-Flip and IAPs.[17]

Many tumour cells, including hepatoma cells,[18,19] exhibit resistance to the proapoptotic effect of TRAIL,[20] but their sensitivity to TRAIL can be increased by combining TRAIL with many compounds, such as anticancer cytotoxic drugs,[21] proteasome inhibitors[22,23] and HDAC inhibitors.[18,19,24,25] These findings have permitted to elaborate several new therapeutical strategies.[26]

We have previously demonstrated that various HDACIs are effective in inducing apoptosis in HepG2 cells.[27] Because little is known[18,19] about the sensitization to TRAIL induced by HDACIs in HCC cells, we have performed a research on this subject. Between the different HDACIs, we have employed for our experiments the suberoylanilide hydroxamic acid (SAHA), because this compound strongly sensitises HCC cells to TRAIL but exhibits a very low toxicity on primary human hepatocytes (PHH). In addition SAHA has shown promising clinical activity against various solid tumours at doses that have been well tolerated by patients.[28]

In this paper we demonstrate that SAHA sensitised HCC cells to TRAIL by inducing up-regulation of DR5 and down-regulation of c-Flip. Both these events led to the activation of TRAIL-DISC and caspase-8 with induction of apoptosis. The consequent activation of caspase-3 provoked cleavage of both NF-kB and Akt, which contributed to the strong apoptotic activity of TRAIL.

2. Materials and methods

2.1. Chemicals and reagents

TRAIL signalling has been activated using a soluble human recombinant TRAIL/APO2L, containing the residues of amino acids from 114 to 281 of natural TRAIL. This compound was purchased from Pepro Tech. (EC Ltd., London, UK) and is reported in this paper as "TRAIL". z-VAD-fmk and DEVD-cho were purchased from Promega (Milan, Italy), IEDT-fmk from Clontech (Palo Alto, CA), z-LEHD-fmk from Imgenex (San Diego, CA). All the other compounds were purchased from Sigma (St. Louis, MO). SAHA was kindly provided by Italfarmaco S.p.a. (Milan, Italy). Stock solutions of SAHA were dissolved in DMSO and diluted in culture medium.

In each experimental condition, DMSO never exceeded 0.04%, a percentage which was not toxic and did not interfere with cell growth.

2.2. Cell cultures, cell viability and cell death assay

HepG2 cells, obtained from "Istituto Scientifico Tumori" (Genoa, Italy), Hep3B and SK-Hep1 cells from the European Collection of Animal Cell Cultures (ECACC, Health Protection Agency, Porton Down, Wiltshire, UK) and PHH, from Cambrex Bio Science (Walkersville, MD), were cultured as described.[29,30] After plating either on 96-well plates or 100-mm culture dishes, cells were allowed to adhere overnight and then treated with chemicals or vehicle only. Cell viability was determined by the MTT quantitative colorimetric assay as described.[31]

Apoptotic cells were detected by flow cytometry analysis using the annexinV-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen; San Diego, CA), according to the manufacturer's instructions. In order to ascertain the presence of condensed chromatin and apoptotic bodies, cells were fixed in 3:1 methanol/acetic acid and incubated for 30 min with Hoechst 33258. After washing in PBS nuclear morphology was observed under a fluorescence microscope.

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analysed as described.[31]

2.3. Western blotting analysis

Cell lysates were prepared as reported.[32] Protein samples (30 μg/lane) were subjected to SDS polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane for detection with specific antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protein bands were developed by Enhanced Chemiluminescence (Pierce, Rockford, IL) and their intensity was quantified by using Quantity One quantification analysis software (Bio-Rad, Hercules, CA). The correct protein loading was ascertained by means of both red Ponceau staining and immunoblotting for β-actin.