CERAMIDE IS NOT INVOLVED IN NUCLEAR TRANSLOCATION OF NF-κB IN L5178Y SUBLINES

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Sphingolipids have emerged recently as regulators of processes controlled by extracellular agents. It was found that the stimulation of cell surface receptors, or oxidative stress, due to the production of reactive oxygen species in cells exposed to ionizing radiation, generate ceramide, a "death messenger", since it is a signal for apoptotic death.

A question of both practical and theoretical interest is whether radiation-induced apoptosis is initiated by a signal generated in the cell membrane or in the nucleus. Fragmentation of nuclear DNA could be produced by a direct interaction with X rays or with the reactive oxygen intermediates generated within the cell by ionizing radiation. Primary DNA lesions have been considered as signals that trigger the apoptotic response [1]; consistently, activation of ceramide synthase (another enzyme involved in ceramide generation) is p53-dependent [2]. Other investigations provide evidence that the apoptotic signalling can also be generated by an interaction of ionizing radiation with the plasma membrane. This interaction initiates sphingomyelin hydrolysis to ceramide [3].

The use of cell permeable synthetic ceramides has shown that protein kinases stimulated by ceramides directly regulate the function of transcription factors, mainly the nuclear factor κB (NF-κB). These protein kinases are important for a coordinated regulation of gene expression in the stressed cell. NF-κB is involved in the transcriptional control of genes that regulate cell death, such as c-myc and p53 [4]. The predominant form of NF-κB in unstimulated cells occurs in the cytoplasm as a heterodimer of two proteins, p50 and p65, complexed to an inhibitory subunit called I-κB; the latter prevents migration of the heterodimer to the nucleus and its binding to DNA. Upon cell stimulation (e.g. by cytokines or ionizing radiation) this inhibitory subunit becomes phosphorylated and dissociates from the NF-κB heterodimer. The released heterodimer (p50/p65) migrates to the nucleus where it can bind to DNA and stimulate transcription. Interestingly, ceramide has been shown to induce the translocation and activation of NF-κB. It possibly takes place through the phosphorylation of the cytosolic I-κB inhibitor by protein kinase Cξ [5], thereby providing a link to integrate the sphingomyelin cycle with NF-κB-dependent pathways.

Differential activation of NF-κB reflects the antioxidant defence efficiency in L5178Y (LY) murine lymphoma sublines, LY-R and LY-S, that differ in sensitivity to ionizing radiation and oxidants [6,7]. Therefore, it has been of interest to examine, whether ceramide is involved in translocation and activation of NF-κB in this pair of sublines. We used two synthetic ceramides, C2 (N-acetyl-D-sphingosine) and C6 (N-hexanoyl-D-sphingosine), as well as PMA, phorbol ester, which is an efficient activator of NF-κB in LY-R cells and a weaker activator - in LY-S cells (paper submitted). Fig.1 presents western blots of nuclear extracts from LY sublines. As shown, only PMA gives a clear activation of the transcription factor. The two ceramides tested show no activatory effect. Therefore, other pathways are employed in LY cells for activation of NF-κB. A recent report indicates that in the irradiated cell the pathway is atm-dependent (*ataxia telaniectasia mutated*) [8].

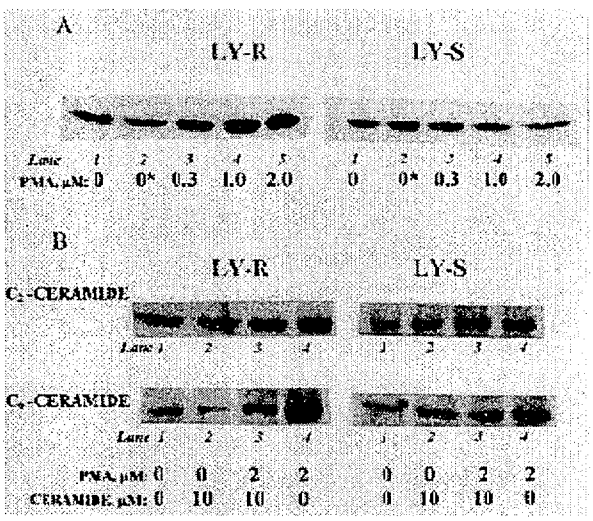

Fig.1. Activation of NFκB in LY sublines, as judged form the nuclear translocation of the p65 subunit. Western blots shown: A - after treatment (1 h, 37°C) with various concentrations of PMA alone (lane 1 - control, lane 2 - control with 0.3% acetone, solute used for PMA); B - after PMA and/or ceramide treatment; C2 or C6 ceramide (see text) applied at 10 μM concentration for 1 h at 37°C alone or preceding 1 h (37°C) incubation with PMA 2 μM (lane 1 - control; lane 2 - ceramide alone; lane 3 - PMA followed by ceramide; lane 4 - PMA alone). All nuclear extract samples from correspondingly treated cells contained 30 μg of protein per lane. Note a more pronounced effect of PMA on nuclear translocation of NFκB in LY-R than in LY-S cells and lack of effect of ceramide.

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INDUCTION OF DNA BREAKAGE IN X-IRRADIATED NUCLEOIDS SELECTIVELY STRIPPED OF NUCLEAR PROTEINS IN TWO MOUSE LYMPHOMA CELL LINES DIFFERING IN RADIOSENSITIVITY

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DNA in the nucleus of eukaryotic cells is tightly associated with histones and other nuclear proteins and folded into a higher order chromatin structure that is anchored to the nuclear matrix. Chromatin proteins are important not only for the maintenance of chromatin structure but also may protect DNA against exogenous damage. Regions of chromatin of more open conformation, like transcriptionally active DNA, are more susceptible to ionizing radiation than inactive regions [1,2]. Proteins may also protect DNA against radiation-generated free radicals, or may serve as a source of reducing equivalents for chemical repair of DNA radicals.

To examine the role of nuclear proteins in protection of DNA against ionizing radiation and their contribution to the radiation sensitivity, an alkaline version of comet assay, which detects overall DNA damage i.e. DNA breaks and alkali-labile sites, was used to estimate DNA damage [3]. The cellular model consisted of two L5178Y (LY) mouse lymphoma cell lines, LY-S and LY-R, differing in sensitivity to ionizing radiation; D_0 values of survival curves (the dose required to reduce the surviving fraction by the factor e^{-1}) are 0.5 Gy and 1 Gy, respectively. Sequential removal of nuclear proteins by an extraction with different concentrations of NaCl resulted in an increase of DNA damage in LY-R nucleoids. Removal of histone Hl and a part of non-histone proteins (NHP's) (0.8 M NaCl) caused 1.1 fold increase in the mean DNA content in the comet tails of the irradiated nucleoids, as compared to nucleoids treated with 0.14 M NaCl ($p<0.01$). Total removal of histones and NHP's (2.5 M NaCl) resulted in 1.25-fold increase in DNA damage of irradiated nucleoids ($p<0.01$, Fig.1). In contrast, in the radiation sensitive LY-S cell line, depletion of nuclear protein practically did not affect DNA damage (Fig.1).

In control (non-irradiated) cells the sequential selective removal of chromatin proteins from the nucleoids did not result in any significant changes in DNA mobility. However, we found significantly more DNA in the comet tail of control LY-R cells than in LY-S cells ($p<0.05$). This phenomenon may reflect a higher level of endogenously generated DNA damage in LY-R cells due to the higher steady-state transition metal ion content (for discussion see [4]) and has repeatedly been observed by us in the comet assay with LY cells. Another explanation of this phenomenon may be a different chromatin organisation in LY sublines, previously proposed in view of the different supercoiling properties of nucleoids from LY cells [5]. Nevertheless, the initial DNA damage induced in unextracted chromatin with 1.5 Gy of X radiation was similar in the two cell lines. A similar level of initial DNA breakage was also found previously in non-exposed cells [6].

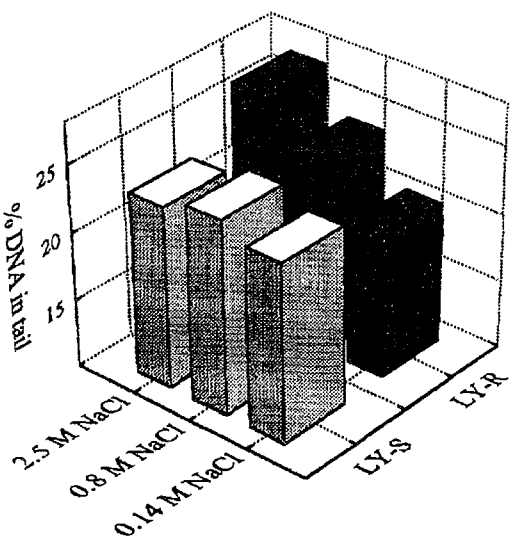

Fig.1. DNA damage in protein-stripped nucleoids of L5178Y cell lines expressed as a percentage of DNA in the comet "tail". Data represent mean values for 50 comets after subtraction of the mean value for 50 control comets.

Although there is no doubt that the main cause of LY-S cells' sensitivity to ionizing radiation is a defect in the repair of DSB's, our data support the concept that chromatin organisation may contribute to the cellular susceptibility to DNA damaging agents.

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