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ADDITIONAL EVIDENCE FOR THE INVOLVEMENT OF ENDONUCLEASE P23 IN NECROSIS.

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Two major forms of cell death have been described: apoptosis and necrosis. Apoptosis (programmed cell death) is a biochemically well-defined, energy-driven process by which a cell actively destroys itself in response to a variety of signals (Rogalinska, 2002). Necrosis is generally considered to be a passive process in which the cell dies as a result of a ruinous depletion of energy (Ziegler and Groscurth, 2005; Fink and Cookson, 2005). One of the key players in both apoptotic and necrotic responses is poly(ADP-ribose) polymerase-1 (PARP-1) which is a nuclear enzyme that under homeostatic conditions participates in DNA repair. It is activated by DNA strand breaks and catalyzes the ATP-dependent poly(ADP-ribosyl)ation of a variety of nuclear acceptor proteins, including itself (Nguewa et al., 2003). A correlation between poly(ADP-ribosyl)ation and the activity of several Ca^{2+}/Mg^{2+}-dependent endonucleases and the apoptotic endonuclease CAD has been documented (Yakovlev et al., 2000; Boulares et al., 2002; West et al., 2005). Previously, we characterized a 23 kD endonuclease (p23) that is associated with the rat hepatocyte nuclear matrix (Grdović and Poznanović, 2003; Grdović et al., 2005) and presented evidence for its participation in necrotic cell death induced by cryogenic temperatures (Grdović et al., in press). In the present paper, we examined whether poly(ADP-ribosyl)ation affects the activity of endonuclease p23.

Nuclei isolated from cryopreserved liver of adult male Wistar rats served as a source of p23 and PARP-1 activity. Isolated nuclei were subjected to *in vitro* poly(ADP-ribosyl)ation for 60 min at 30°C in a reaction mixture containing 100 µg of nuclear proteins, 40 mM Tris-HCl (7.8), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, and 400 µM NAD as a substrate. After incubation, the nuclei were subjected to endogenous nuclease digestion and subsequent extraction steps in order to isolate the nuclear matrix (Poznanović et al., 1996). Activity of P23 was detected by activity gel analysis (Rauch et al., 1997). *In vitro* poly(ADP-ribosyl)ation was verified by Western immunoblot analyses of nuclear matrix proteins with PARP-1 antibody. The presence of a high-molecular weight smear above the full length protein (116 kD) served as evidence of *in vitro* poly(ADP-ribosyl)ation (Fig. 1A, lane 3).

Incubation of nuclei under conditions favoring poly(ADP-ribosyl)ation completely abolished p23 activity from the subsequently isolated nuclear matrix (Fig. 1B, lane 2). However, in nuclei incubated under conditions in which poly(ADP-ribosyl)ation was inhibited, either due to the presence of the PARP-1 inhibitor 3AB (Fig. 1B, lane 3) or to the absence of NAD, the substrate for poly(ADP-ribosyl)ation (lane 4), p23 activity was not observed in the nuclear matrix, effectively ruling out both direct and indirect influence of poly(ADP-ribosyl)ation on p23 inhibition. The absence of p23 activity in the nuclear matrix suggests that the enzyme was released from the nuclear matrix during the incubation step. The supernatants obtained after incubation of nuclei in reaction mixtures either with or without NAD and in the presence of 3AB were tested for the presence of p23 activity (Fig. 1B, lanes 5, 7, and 6, respectively). Activity gel analysis confirmed the dissociation of p23 from the insoluble nuclear fraction and also revealed that poly(ADP-ribosyl)ation did not inhibit p23. These results were further supported by an experiment in which the nuclear matrix was subjected to *in vitro* poly(ADP-ribosyl)ation in the presence of recombinant PARP (10 U/µl of reaction mixture). Activity gel analysis clearly showed that p23 activity was not altered in any way as a result of PARP-1 activity (Fig. 1B, lane 8).

It is known that PARP-1 functions as a molecular switch between apoptotic and necrotic modes of cell death (Los et al., 2002). Overactivation of PARP-1 depletes intracellular ATP levels and irreversibly leads to cell death with a necrotic morphology. Since apoptosis is an energy requiring process, a crucial step in the apoptotic pathway is the preservation of cellular ATP by prevention of its depletion through excessive PARP activation. One of the earliest events in apoptosis is the cleavage and inactivation of PARP-1 by caspases 3 and 7 (Cohen, 1997). The consequence of PARP-1 cleavage could be the release of certain nuclear proteins from poly(ADP-ribosyl)ation-induced inhibition (Yakovlev et al., 1999). Thus, it is not surprising that endonucleases presumed to mediate apoptotic internucleosomal DNA degradation are inhibited by poly(ADP-ribosyl)ation (Yakovlev et al., 2000; Boulares et al., 2002; West et al., 2005). Therefore, the cleavage of PARP-1 during early apoptosis could lead to activation of endonuclease(s) that are required for DNA degradation. The necrotic

process is occasionally characterized by PARP-1 overactivation (L e i s t et al., 1997; H a and S n y d e r, 1999). Thus, the inhibition of an endonuclease that mediates necrotic DNA fragmentation by poly(ADP-ribosyl)ation would be unsustainable. Here we show that p23 was not inhibited by PARP-1 activation.

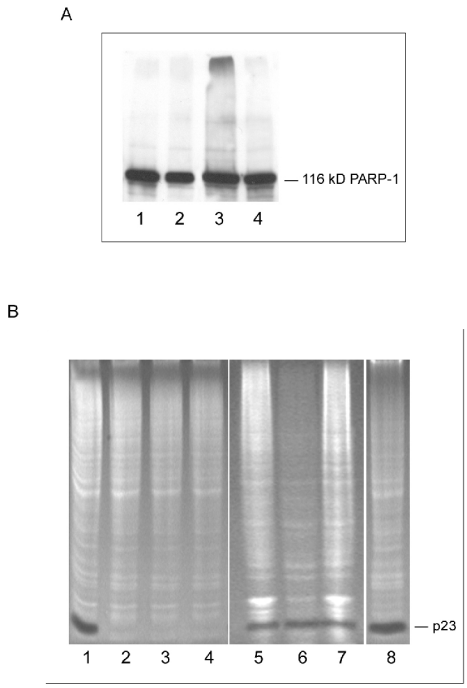

Fig. 1. The effect of poly(ADP-ribosyl)ation on p23 endonuclease. A - A - Western immunoblot analyses of nuclear matrix proteins with PARP-1 antibody. Nuclear matrices were isolated from untreated nuclei (lane 1) or nuclei that were incubated for 60 min at 30°C in poly(ADP-ribosyl)ation reaction mixture with NAD (lane 3), without NAD (lane 2), or in the presence of both NAD and 3AB (lane 4).
B - Detection of p23 activity by activity gel analysis. Nuclear matrix proteins isolated from untreated nuclei (lane 1) and nuclei that were incubated in poly(ADP-ribosyl)ation reaction mixture with NAD (lane 2), without NAD (lane 4), or in the presence of both NAD and 3AB (lane 3). Supernatant protein fraction obtained after poly(ADP-ribosyl)ation of nuclei in the presence of NAD (lane 5), both NAD and 3AB (lane 6), or without NAD (lane7). In vitro poly(ADP-ribosyl)ation of nuclear matrix proteins in the presence of recombinant PARP-1 (10U/µl of reaction mixture) (lane 8).

As p23 is bound to the nuclear matrix through disulfide linkages (G r d o v i ć et al., 2005), its release from the nuclear matrix was the result of reduction of disulfide bonds by β-mercaptoethanol in the incubation buffer. The finding that p23 was active in the soluble fraction regardless of PARP-1 activity is in agreement with our previous observation that p23 is activated during cryonecrosis in rat liver *in vivo* and with the assumption that the observed DNA degradation to high-molecular-weight fragments resulted from its activation (G r d o v i ć et al., in press). Since the biochemical events that underlie necrotic cell death are still mostly unclear, the identification of the endonuclease that mediates necrotic DNA degradation would greatly contribute towards our understanding of the necrotic process.

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