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FOREWORD

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INTRODUCTION

This research effort is directed at subobjective 2 as defined in the recent shock trauma mediators review memo from the U.S. Army Institute of Surgical Research. Specifically, we aim at the prevention of secondary damage after hemorrhage by temporizing fundamental physiological or biochemical processes leading to cell death and organ failure. For this purpose, increased expression of the inducible heat shock protein 70 (HSP70i) will be employed. HSP70i works as a chaperone attaching to short hydrophobic peptide sequences as they are exposed in ischemic cells in which proteins undergo denaturation. By the association of HSP70 with such protein sequences, further protein aggregation and cell damage is prevented. This leads to a faster recovery of cell function after the ischemic or hemorrhagic episode is reversed. Our results which are further summarized in the body indicate that overexpression of HSP70 in transgenic muscle-type cell lines lead to increased protection of such cells against ischemic damage. In addition, recent work in transgenic mice in which the HSP70 protein was overexpressed in heart, brain and skeletal muscle show, especially for studies in the heart, that the ischemic myocardium shows enhanced protection against ischemia related injury. In discussing the studies with Col. Smallridge, M.D., specific areas of interest at Walter Reed Army Hospital were identified. They relate to the prevention of ischemic hemorrhagic injury resulting in brain, kidney and gut. For this purpose, additional transgenic animals are bred leading to increased expression of heat shock proteins in these organs.

BODY

In recent studies which are published in the Journal of Clinical Investigation (1994;93:759-767, copy attached), we used the myocytic cell line H9c2 cells. These cells were initially derived from the fetal myocardium of the rat. These cells grow initially as myoblasts then fuse into myotubes. The cells were engineered using a stable transfection protocol to overexpress the human HSP70i gene which was kindly provided by Dr. Richard Morimoto using a neomycin selection approach. Exposing these cells to simulated ischemia which include hypoxia (oxygen tension of less than 0.04%) and the absence of glucose in the medium. After the episode of simulated ischemia, the incubation chamber is opened and cells were re-exposed to oxygen. In these cells, colony survival was determined by standard methods. In addition, we determined LDH release and the intactness of mitochondria using the vital dye rhodamine 123. As indicated in this paper, cells overexpressing the human inducible HSP70 showed a significant survival advantage after simulated ischemia using the colony survival assay in addition a much lower amount of LDH was released from these cells and mitochondria show less damage. The studies, therefore, show that excess expression of HSP70 leads to increased protection against an ischemic damage.

To confirm the increased protection which is mediated by HSP70 in cell culture in an *in vivo* system, we took the following approach. We produced transgenic mice which overexpress a HSP70 transgene. In the transgene, we used the human cytomegalovirus enhancer linked to a chicken β -actin promoter and the chicken β -actin intron. In the multiple cloning site following the chicken β -actin intron, we placed a full length cDNA coding for the rat HSP70i. Several lines of transgenic mice were obtained expressing the heat shock proteins at a very high level (Figure 1). A Western blot which is attached shows that high

levels of the rat HSP70i transgene is expressed in heart, skeletal muscle, and brain in the heterozygous lines of these mice. We are currently breeding homozygous lines in which expression in kidney and gut will be evaluated. Hearts were obtained from control mice and transgene positive mice and an isolated Langendorf heart preparation was established. These hearts were then submitted to global ischemia for 20 minutes and then reperfusion was started. At different time points after reperfusion, the contractile performance of these hearts were determined by attaching a force transducer to the apex of the mouse hearts. In addition, infarct size was quantitated using a tetrazolium based technique. Our results which are currently submitted for publication to the Journal of Clinical Investigation indicate that in the hearts obtained from transgenic mice overexpressing the heat shock protein functional recovery was improved by 30% in comparison to control hearts. In addition, infarct size was reduced by 40%. Furthermore, the liberation of creatine kinase was markedly diminished. These results, therefore, were similar to those described in cell lines above that expression of the inducible heat shock protein has a marked protective effect against ischemia. In addition to the studies described above, we have cloned the inducible HSP70 into an adenovirus vector (Figure 2). We are currently in the process of preparing for studies in rabbits. In these studies, the adenovirus coding for HSP70 will be infused into the rabbit coronary and infarct sizing will be obtained in rabbits infused with the adenovirus positive for the HSP70 transgene and adenovirus coding only for β -galactosidase. This adenovirus will be available to Dr. Smallridge's group in the future for specific studies which are of their interest.

CONCLUSION

In summary, we could show in a stable striated muscle cell line that expression of the human inducible HSP70 exerts a protective effect against simulated ischemic damage under cell culture conditions. In addition, we could demonstrate that a protective effect against ischemic damage also occurs under *in vivo* conditions in mice in which the rat inducible heat shock protein is expressed in the heart. These mice also express excess amounts of HSPs in brain and skeletal muscle and will be available for investigators at the Walter Reed Army Hospital to determine if a protective effect can be elicited in these organs. Furthermore, we have constructed an adenovirus expressing the inducible heat shock protein. This adenovirus will be available to determine in cell lines of interest to investigators at the Army Hospital like the 431 cell line and GH3 cell lines if a protective effect of heat shock proteins can also be elicited in these cells. We, therefore, think we have been highly productive in our research effort demonstrating that increased expression of heat shock proteins can have a protective effect against ischemic damage. By using adenovirus or similar particle in the future it will be possible to harness the protective effects of specific heat shock proteins using a gene therapy based approach.

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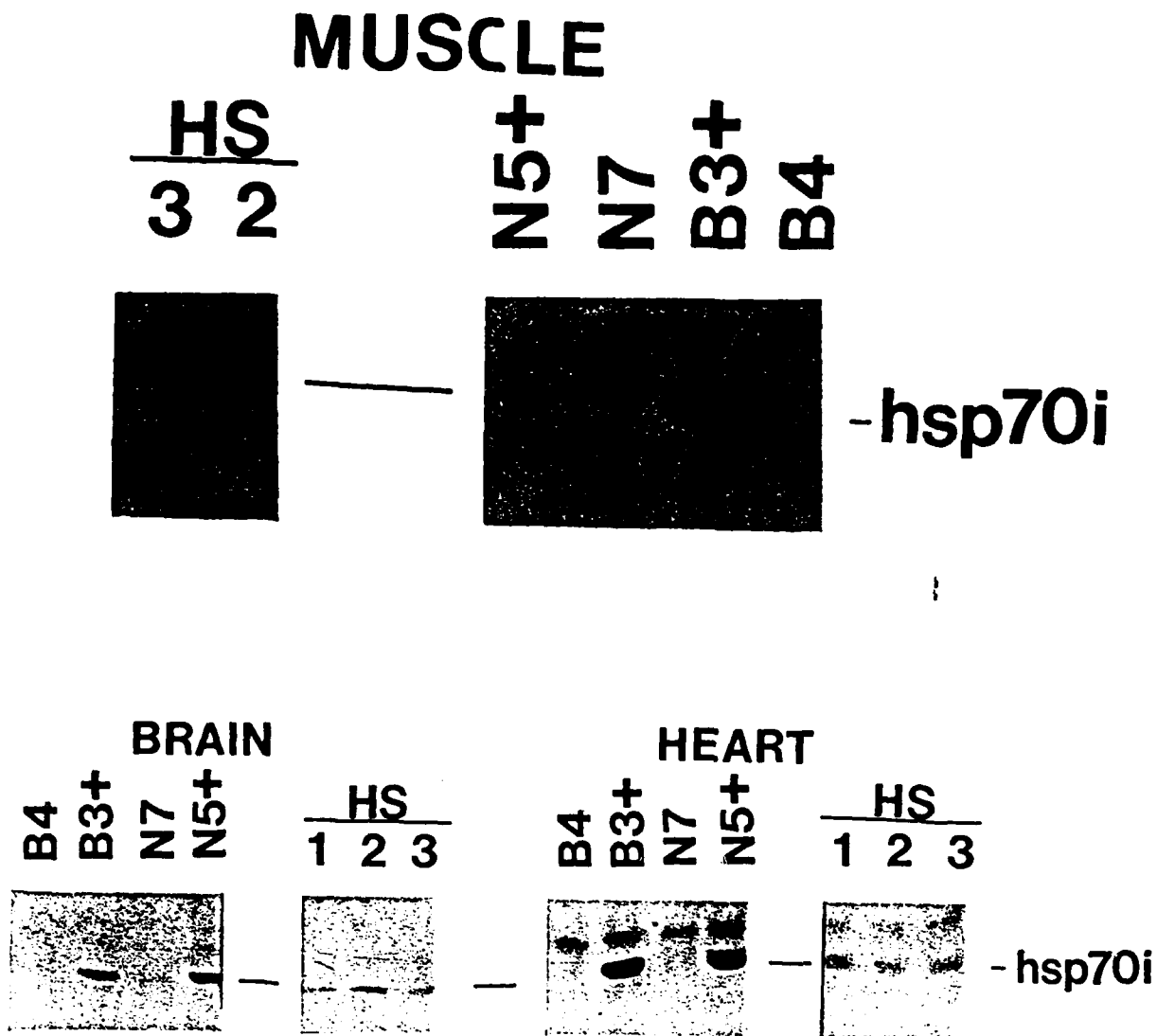


FIGURE 1: Western blot of protein extracts from heart, muscle and brain tissue of negative (Br and N7) and positive (B3 and N5) transgenic mice and non-transgenic mice that were heat shocked (HS 1,2,3) and subsequently recovered for 8-24 hours. Blot was reacted with monoclonal antibody raised against the HSP70 which binds only to the inducible forms of HSP70 (C-92F3A-5, StressGen). Transgenic mice were identified by Southern blot analysis.

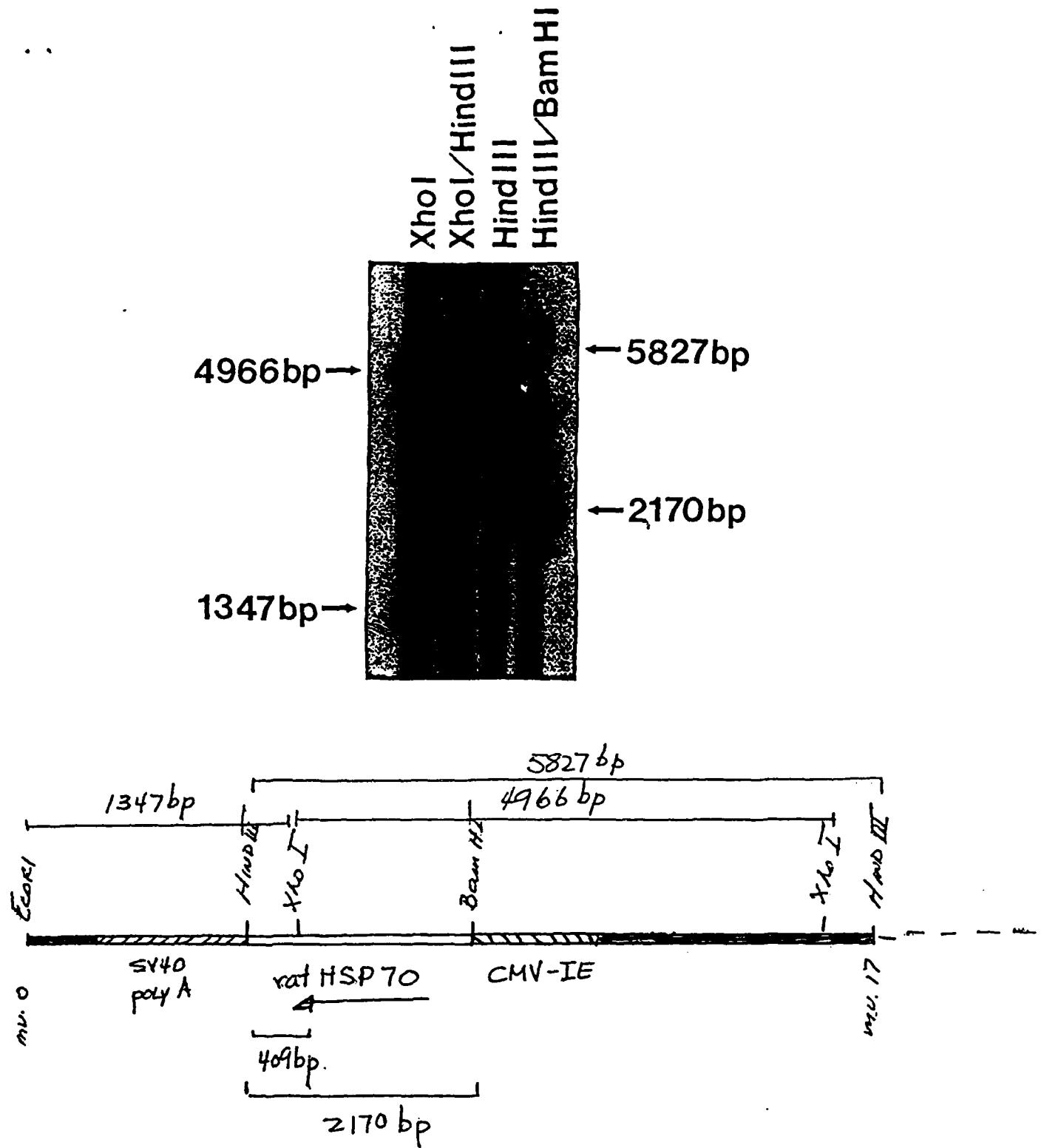


FIGURE 2: Southern blot of adenovirus vector (AdV70) which carries the rat inducible HSP70 coding region. DNA was prepared by the Hirt method from infected 293 cells. AdV70 DNA was digested with several restriction enzymes to confirm the presence of rat HSP70 in the adenovirus vector. The Southern blot was hybridized with the complete coding region of the rat HSP70. Bands in the Southern show the presence of the rat HSP70 in the adenovirus (Hind III/Bam HI digest, 2170 bp band = size of coding region of rHSP70). Band at 1347 bp in lane digested with Xho I shows that orientation of rat HSP70 is in the proper direction.