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We investigated programmed cell death (apoptosis) in human umbilical vein endothelial cells (HUVEC) and hepatoma cells subjected to hypoxia. We examined apoptosis by DNA ladder, 3'-end labeling of DNA and morphology. Hypoxia induced apoptosis in both HUVEC and RH7777 hepatoma cells but with a different time course for each cell type. The cell line RH 7777 were apoptotic within 12 hours of exposure to hypoxia; whereas, the HUVEC required at least 36 hours before they began to be apoptotic. We looked for expression of the Bcl-2 family of cyto-protective (A1, Bcl-2, and Bcl-xL) molecules by either Northern or Western analysis and found no increased expression. We conclude that these molecules are not responsible for the cyto-protection seen in HUVEC during the first 24 to 36 hours of hypoxia.

INTERDEPARTMENTAL

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February 24, 1998

TO: Office of Naval Research

FROM: Robert K. Winn, Ph.D.
Research Professor

RE: Progress Report

Please find my final report enclosed.

FINAL REPORT:

GRANT #: N00014-95-1-0784

PRINCIPAL INVESTIGATOR: Robert K. Winn

INSTITUTION: University of Washington

GRANT TITLE: The Role of Apoptosis in Hypoxic Endothelial Cell Injury.

AWARD PERIOD: 1 May 1995 - 30 April 1996

OBJECTIVE: To investigate the regulation of programmed cell death (apoptosis) in hypoxia resistant endothelial cells and hypoxia sensitive hepatoma cells. We will examine the cyto-protective role of Bcl-2 homologues in endothelial cells. Likewise, we will examine the ability of ICE and the ICE-like protease CPP32/YAMA to produce apoptosis.

APPROACH: HUVEC were subjected to hypoxia in a controlled atmosphere chamber and apoptosis determined by DNA agarose gel electrophoresis, 3'-DNA end labeling and morphology.

ACCOMPLISHMENTS: Human umbilical vein endothelial cells (HUVEC) were exposed to normoxia or hypoxia for up to 48 hours. Apoptosis in HUVEC was induced when they were exposed to hypoxic for 48 but not 24 or 36 hours. Apoptosis was evaluated by DNA fragmentation (ladder), 3'-end labeling (TUNEL) and by cellular morphology. A similar experiment used the hepatoma cell line MH 7777 to evaluate when they become apoptotic in an hypoxic environment. Cells were cultured in standard culture medium in either normoxic or hypoxic environments.

DNA fragmentation was determined by agarose gel electrophoresis from the typical ladder pattern. HUVEC were in the hypoxic environment for 24,36 or 48 hours, whereas, the hepatoma cells were hypoxic for 4, 12, or 24 hours. Electrophoresis of DNA extracted from hypoxic endothelial cells after 48 hours showed the typical ladder pattern but not when extracted after 24 or 36 hours. The HUVEC contrast with MH7777 cells where apoptosis was initiated by 12 hours of hypoxia. The DNA ladder after 12 hours of hypoxic suggests that these cells are more sensitive to hypoxia induced apoptosis than HUVEC.

The short sequences of DNA that produce ladders on electrophoresis of apoptotic cells yields a significant increase in the number of 3' ends. Labeling the 3' ends with digoxigenen using terminal deoxynucleotidyl transferase (TdT) allows detection of apoptosis by flow cytometry. Double staining with propidium iodide (PI), a DNA stain and FITC-labeled anti-digoxigenen allows the display of normal and

apoptotic cells simultaneously. The percent apoptotic cells determined as the ratio of FITC positive cells relative to PI positive cells. We estimated apoptosis from 3 separate experiments to be 1.0±0.9%, 2.2±1.2%, 6.1±4.5% and 48.1±23.2% for 24 hours normoxia, 24 hours hypoxia, 48 hours normoxia and 48 hours hypoxia, respectively. Significant apoptosis was induced only after 48 hours.

Apoptosis was also determined by morphology. Differential cell counts (i.e., number of apoptotic cells relative to total cells) were completed by manually counting. This resulted in 8.0±6.1%, 12.9±11.5%, 10.6±2.9%, and 52.0±14.7% for 24 hours normoxia, 24 hours hypoxia, 48 hours normoxia and 48 hours hypoxia, respectively. Percent apoptosis measured by cytometry depends on assignment of normal FITC fluorescence (i.e., non-apoptotic), thus a quantitative difference is to be expected between apoptosis determined by cytometry and morphology.

Total RNA was extracted from HUVEC incubated for 24, 36 or 48 hours, subjected to electrophoresis on a formaldehyde-agarose gel and transferred to a nylon membrane. We probed the blot with a radio-labeled probe that recognizes human A1 and found very little expression of A1 mRNA at 24, 36 or 48 hour in both the control cells and hypoxic cells. Actin mRNA was approximately equal in these samples.

CONCLUSIONS: Hypoxia induces apoptosis in HUVEC after exposure for 36 hours and this represents a tolerance to hypoxia. The cell line RH 7777 are apoptotic within 12 hours of exposure to hypoxia.

SIGNIFICANCE: Bcl-2 homologues may provide some protection from apoptosis due to hypoxia; however, northern blot analysis probing for A1 suggest that this molecule is not responsible for cyto-protection.

PATENT INFORMATION: None.

AWARD INFORMATION: None.

PUBLICATIONS AND ABSTRACTS:

1. Cornejo, C.J., J.M. Harlan, and R.K. Winn. Human Umbilical Vein Endothelial Cells (HUVEC) Are Resistant to Hypoxia-Induced Apoptosis. FASEB J. A45, 1996.

HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC) ARE RESISTANT TO HYPOXIA-INDUCED APOPTOSIS

Carol J. Cornejo, John M. Harlan, and Robert K. Winn

We examined whether HUVEC cultures were resistant to hypoxia-induced apoptosis compared with a hepatoma cell line, (RH7777). HUVEC were placed in a hypoxic chamber (95% N2, 5% CO2) for 24 or 48 hr and RH7777 were placed in the hypoxic chamber for 4 or 12 hr. Cells kept in room air with 5% CO2 for the same time periods were used as normoxic controls. The presence of apoptosis was determined by looking for the distinct 180-200 bp ladder on DNA gel electrophoresis and by labelling the 3' ends of DNA and detecting this label using flow cytometry. Data were analyzed using Student's t test. HUVEC were found to have a DNA ladder at 48 hr of hypoxia but not at 24 hr. RH7777 had a DNA ladder at 12 hr of hypoxia but not at 4 hr. This correlated with the number of apoptotic cells seen by cytometry. There were significantly more apoptotic HUVEC at 48 hr of hypoxia compared to 48 hr of normoxia ($48 \pm 23\%$ vs $4 \pm 4\%$) ($p<.05$). The number of apoptotic cells at 24 hr was not different when hypoxia was compared to normoxia. There were significantly more apoptotic RH7777 at 12 hr of hypoxia compared to 12 hr of normoxia ($63 \pm 4\%$ vs $2 \pm .5\%$) ($p<0.05$). The number of apoptotic RH7777 cells at 4 hr was not different between hypoxia and normoxia. In summary, HUVEC require 48 hr of hypoxia to undergo apoptosis while RH7777 require only 12 hr of hypoxia. Therefore, we conclude that HUVEC are more resistant to hypoxia-induced apoptosis than RH7777. We are currently examining the role of the bcl-2 homologues in providing protection against hypoxia-induced apoptosis. (Supported by Office of Naval Research Grant #N00014-95-1-0784).

Annual Progress Report:

Grant #: N00014-96-1-285

Principal Investigator: Robert K. Winn

Institution: University of Washington

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Grant Title: The Role of Apoptosis in Hypoxic Endothelial Cell Injury.

Reporting Period: 1 June 1995 - 31 May 1996

Award Period: 1 May 1995 - 30 April 1999

Objective: To investigate the regulation of programmed cell death (apoptosis) in hypoxia resistant endothelial cells and hypoxia sensitive hepatoma cells. We will examine the cyto-protective role of Bcl-2 homologues in endothelial cells. Likewise, we will examine the ability of ICE and the ICE-like protease CPP32/YAMA to produce apoptosis.

Approach: Expression of Bcl-2, Mcl-1, A1, and Bax will be determined by Northern and/or Western analysis. The function of these molecules will be examined by introduction of antisense oligonucleotides. ICE and CPP32/YAMA will be examined in a cell-free system. Function of the proteases will be determined by blocking with peptide based inhibitors.

Accomplishments: Human umbilical vein endothelial cells (HUVEC) were exposed to normoxia or hypoxia for up to 48 hours. Apoptosis in HUVEC was induced when they were exposed to hypoxic for 48 but not 24 or 36 hours. Apoptosis was evaluated by DNA fragmentation (ladder), 3'-end labeling (TUNEL) and by cellular morphology. A similar experiment used the hepatoma cell line MH 7777 to evaluate when they become apoptotic in an hypoxic environment. Cells were cultured in standard culture medium in either normoxic or hypoxic environments.

DNA fragmentation was determined by agarose gel electrophoresis from the typical ladder pattern. HUVEC were in the hypoxic environment for 24,36 or 48 hours, whereas, the hepatoma cells were hypoxic for 4, 12, or 24 hours. Electrophoresis of DNA extracted from hypoxic endothelial cells after 48 hours showed the typical ladder pattern but not when extracted after 24 or 36 hours. The HUVEC contrast with MH7777 cells where apoptosis was initiated by 12 hours of hypoxia. The DNA ladder after 12 hours of hypoxic suggests that these cells are more sensitive to hypoxia induced apoptosis than HUVEC.

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Significance: Bcl-2 homologues may provide some protection from apoptosis due to hypoxia at 24 and 36 hours after initiating hypoxia. By 48 hours of hypoxia A1 mRNA expression was reduced and apoptosis occurred. Again consistent with A1 providing protection to this environmental stress. The suggested protection by A1 assumes that A1 protein is expressed following production of mRNA.

Work Plan (Next 12 Months): We will continue examination of mRNA and protein expression of Bcl-2 family of cyto protective molecules in both endothelial cells and hepatoma cells. Also, we will also begin investigations into the role of the interleukin-1 converting enzyme family of proteases in hypoxia induced apoptosis.

Publications and Abstracts (last 12 months):

1. Cornejo, C.J., J.M. Harlan, and R.K. Winn. Human Umbilical Vein Endothelial Cells (HUVEC) Are Resistant to Hypoxia-Induced Apoptosis. FASEB J. A45, 1996.