Role of Apoptosis Following Cerebral Hypoxia-Ischemia in Immature and Older Rats

By

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A Thesis
Submitted to the Faculty of Graduate Studies in
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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RANJINDER S. SIDHU

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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This thesis is dedicated to my family
and friends for their
support and understanding

Abstract

Cell death following cerebral hypoxia-ischemia occurs by possibly two distinct processes with characteristic morphologies. Traditionally, cell death was considered to occur by the uncontrolled action of degradative enzymes on a lethally injured cell. Evidence now suggests that death may occur through apoptosis, a mode of cell death with the characteristics of programmed cell death that occurs naturally during development. During apoptosis members of the family of cysteine aspartyl-specific proteases (caspases) become activated and degrade essential components of the cellular machinery allowing the elimination of unwanted cells. In the present study, we sought to investigate the age dependence of apoptosis during cerebral hypoxia-ischemia and the effectiveness of a specific caspase-3 inhibitor. To determine the role of apoptosis in mature and immature brain following an episode of cerebral hypoxia-ischemia, cell types were examined under light microscopy and were quantified morphologically. Evidence of punctate chromatin condensation, indicative of apoptosis, was greater in immature brain than in older brain. Role of caspase-3 was examined by measuring caspase-3 activity and caspase-3 protein using Western blotting techniques. Caspase-3 activity was significantly increased in animals exposed to hypoxia-ischemia, with a 3-fold greater caspase-3 activity in immature than older brain. In addition active caspase-3 was detectable 18hr post-hypoxia in 4 wk olds and at 4hr and 18hr following hypoxia in 1 wk olds. In the last study, animals were treated with a caspase-3 specific inhibitor in order to determine its effectiveness as a neuroprotective agent. Doses of 1.5-6μg/g body weight of z-DEVD-fmk I.P. were not effective in reducing infarction. The results demonstrate an increased prevalence of an apoptotic type of morphology and an increased caspase-3 activity in young animals suggesting that there may be a greater contribution of apoptosis in immature than older brain at equivalent time points following hypoxia-ischemia.

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"It is a profound and necessary truth that the deep things in science are not found because they are useful; they are found because it was possible to find them." - Robert Oppenheimer.

List of Figures

List of Tables

Table of Contents

Introduction

Preamble

Low oxygen tension (hypoxia) and low blood flow (ischemia) lead to an imbalance of homeostatic cellular mechanisms. The inability to reestablish homeostasis results in widespread cellular death. Depending on the duration and severity of the ischemia sustained (Bonfoco *et al.*, 1995; Ankarcrona, 1995) cell death in the central nervous system is generally thought to progress through one of two distinct mechanisms: i) an active mode that is under genetic control, or ii) by the uncontrolled activation of degradative enzymes. The deliberate suicide of cells, which protects neighboring cells from degradative enzymes, constitutes an evolutionarily altruistic process known as *apoptosis*. This process is mechanistically related but not identical to the programmed cell death that occurs during embyrogenesis. In contrast, cell death by *necrosis*, due to the inability of the cell to cope with the changes in the milieu, is accompanied by an inflammatory response that may affect surrounding cells.

Studies have shown that multicellular organisms such as the nematode (*Caenorhabditis (sp.) elegans*) and the fruit fly (*Drosophila melanogaster*) are excellent tools for the study of programmed cell death (Steller, 1995). In fact, studying these species led to the discovery of many homologous enzymes in the mammalian system (Fernandes-Alnemri *et al.*, 1994). Programmed cell death (PCD) occurs during normal development and is used as an efficient mechanism of eliminating unwanted cells. These unwanted cells may have served a function at

some time during development but are no longer necessary to the organism. Some obvious examples of this process are the regression of the tadpole tail during metamorphosis, the removal of interdigital webs in amniotes, and the regression of the Mullerian duct in male embryos (Raff, 1992; for detailed review of PCD in vertebrates see review by Sanders and Wride, 1995).

A class of genes central to the initiation or implementation of programmed cell death in the nematode *C. elegans* is the *ced* genes. The Ced (cell death abnormal) genes code for proteins involved in regulating programmed cell death. Many of these proteins have homologous mammalian counterparts and have also been implicated in apoptotic cell death. For example, the *ced-9* gene codes for a protein homologous to the *Bcl-2* protein family in mammals. These are either positive or negative regulators of apoptosis. The *ced-3* gene codes for a protein that is a homolog of the human caspase family of proteases. This family of proteases is considered to be responsible for the execution of the apoptotic process (Cohen, 1997).

Detailed ultrastructural (Wyllie *et al.* 1980) and biochemical studies (Wyllie *et al.*, 1984) illustrated the cytological similarities in normal programmed cell death and apoptosis in acute pathological situations. Subsequently, many researchers equated the terms apoptosis and programmed cell death to describe the characteristic set of morphological changes that accompany cell death in many tissues (Farber, 1994). Apoptotic changes include formation of membrane blebs, which is a pinching off of cell organelles or portions of the nucleus enclosed into membrane bound vesicles. These blebs are called apoptotic bodies and are quickly phagocytosed by neighboring cells. Concomitant with these morphological changes are characteristic

biochemical changes. This results in the activation of proteases or calcium- and magnesium-dependent endonucleases responsible for the cleavage of DNA. This DNA cleavage can be resolved by electrophoresis on agarose gels and shown to have a characteristic "ladder" pattern considered typical of programmed cell death and apoptosis. This "DNA ladder" appears as fragments with usually about 180-200 base pairs – the size corresponding to the length of DNA wrapped around the histone octamer in a nucleosome. This indicates that the oligonucleosomal fragments being produced from the chromatin are being cleaved at the linker DNA between the nucleosomes (Schartzman and Cidlowski, 1993). In contrast, the random breakdown of DNA in necrosis appears as a diffuse smear following agarose gel electrophoresis (MacManus *et al.*, 1995; Wyllie *et al.*, 1984).

In cerebral ischemia there is a rapid and transient induction of genes called "immediate early genes." The products of the genes, *fos, jun* and others, are transcription factors that regulate gene expression of many proteins such as nerve growth factor, glial fibrillary acidic protein, and others. Expresssion of the immediate early genes may also be required for in the induction of apoptosis (Matsushima, *et al.*, 1996). Alternatively, a cell fated to undergo apoptosis may not require *de novo* gene expression (Weil, *et al.*, 1996), instead the signal may be extracellular, or originate from other cells such as glial cells.

In contrast, necrosis, which occurs in response to a wide variety of harmful conditions, involves an initial swelling of the cell and its organelles. This is followed by a breakdown of cellular membranes and disintegration of the nuclear structure and cytoplasmic organelles (Chopp and Li, 1996). The cell subsequently shrinks,

condenses and disintegrates. The release of cellular material may eventually lead to an inflammatory response which may aggravate the injury. Inflammation does not occur in apoptotic cell death (Kerr *et al.*, 1972).

The cell death observed subsequent to an episode of ischemia was traditionally thought to be via a necrotic mechanism. However, as discussed below a great deal of evidence has arisen suggesting a portion of both mechanisms exist. It is possible that cells may die by either mechanism depending on the severity of the insult. The existence of apoptotic characteristics following ischemia suggests that the mechanism may also be genetically controlled (Hill, *et al.* 1995; Li *et al.*, 1995; MacManus, *et al.*, 1995). That is, the genes responsible for programmed cell death may also be stimulated by apoptotic cell death. A role for apoptosis in ischemic injury has sparked interest in possible new therapies. Since the apoptotic process is far more controlled than necrosis there is a therapeutic potential of preventing death by regulating apoptosis.

In the newborn, hypoxic-ischemic brain injury has possible grave repercussions on motor, cognitive and behavioral functions. The cellular injury in the newborn is mediated by processes which may differ from those in the adult and may overlap with those of programmed cell death during normal brain development (du Plessis and Johnston, 1997; Tuor *et al.*, 1996). As a result, the therapeutic strategies used with proven efficacy in adults may not be applicable to the immature brain. In addition, it is possible that the essential normal developmental processes may turn hostile under adverse conditions and contribute to cell death following injury. For

this reason, it is essential to clarify the role of apoptosis in immature and older brain as well as determine the protective effect of the inhibitors of apoptosis.

Pathological Consequences of Hypoxia-Ischemia

Role of Caspases in Apoptosis

The consequences of hypoxic-ischemic injury may result from the deregulation of a multitude of cellular and molecular pathways, ultimately leading to cell death. Cell death following hypoxic-ischemic induced damage has traditionally been thought to occur via an irreversible necrotic type of damage. However, evidence in recent years has accumulated that suggests apoptosis may play at least some role in the process of ischemic cell death.

Death of cultured cerebellar granule neurons occurs by either necrosis or apoptosis depending on the toxic stimulus (Du *et al.*, 1997). The family of cysteine aspartyl-specific proteases (*caspases*), appears to be necessary for the execution of the apoptotic program (Chinnaiyan and Dixit, 1996). Currently there are 11 known mammalian members of the caspase family (***Table I***) (Villa *et al.*, 1997) some of which have been implicated in the neuronal death mechanism. In contrast, *C. elegans* has a single caspase encoded by the ced-3 gene.

Table I* Caspase designations and known substrate specificities

New Name	Old Name	Preferred substrate
Caspase-1	ICE	YEVD>YVAD**
Caspase-2	Ich-1	YDVAD
Caspase-3	CPP32, Yama, apopain	DMQD>DEVD***
Caspase-4	ICE_{rel}-II, TX, ICH-2	LEVD>YEVD
Caspase-5	ICE_{rel}-III, TY	Unknown
Caspase-6	Mch2	VEID
Caspase-7	Mch3, ICE-LAP3, CMH-1	DEVD>DMQD
Caspase-8	MACH, FLICE, Mch5	IETD
Caspase-9	ICH-LAP6, Mch6	Unknown
Caspase-10	Mch4	IEAD
Caspase-11	Ich-3	Unknown

*compiled from Alnemri *et al.*, 1996; Nicholson and Thornberry, 1997, Talanian *et al.*, 1997, Villa *et al.*, 1997; Wang *et al.*, 1998;

**YVAD commonly used for caspase-1 activity

***DEVD commonly used for caspase-3 activity

The distinctive feature of the caspase family of proteases is the absolute requirement for an aspartic acid residue in the substrate at the first position in the cleavage site (P_1 position) (Nicholson, 1996). Different members of the caspase family recognize the target protein, with a different P_4-P_1 sequence, with varying

affinity (Nicholson, 1996). Caspase-3, previously known as CPP32, is of particular interest as it is activated rapidly and is responsible for the cleavage of substrates with the sequence (P_4)DXXD(P_1)-G, cleaving such substrates at P_1 (between D and G) (Nicholson, 1996). Caspase-3 was identified and first shown to cleave a site in the DNA binding domain of the nuclear protein poly(ADP-ribose) polymerase (PARP) (Nicholson *et al*, 1995). Other proteins with a DXXD domain known to be cleaved with similar kinetics include DNA-PK_{cs} and 70kDa protein of U1-ribonucleoprotein (U1-70kD). These proteins function in the repair of DNA double strand breaks and splicing of mRNA, respectively (Casciola-Rosen *et al.* 1996). A general implication suggested from these studies is that proteins involved in the maintenance of normal cell function and repair all have the DXXD motif recognized by caspase-3 (Thornberry, 1997). Proteolytic substrates for other caspases during apoptosis were reviewed by Nicholson and Thornberry (1997). In addition, the active site of caspases and ICE-like proteases has a well conserved pentapeptide sequence QACXG, in which X is R for caspase-3 while other caspases have either Q or G (Fernandes-Alnemri, 1994).

Pharmacological modification of caspase activity

The sequence of the triggering mechanism during apoptosis induced by various stimuli is under intense investigation (see Cohen, 1997 for review of stimuli and triggering mechanisms). Cerebral hypoxia-ischemia results in a severe alteration of the cellular homeostatic mechanisms. A major determinant of the degree of damage sustained during ischemia is the level of glucose, the primary cerebral energy

source for immature and mature brain. Glucose is used for the production of adenosine triphosphate (ATP), the substrate of oxidative metabolism. A lack of oxygen induces a switch to anaerobic glycolysis eventually leading to acidosis from the accumulated lactate (Siesjö *et al.*, 1996). In many situations, energy failure could be due to insufficient ATP production, which may be in part the result of mitochondrial failure. This depletion in ATP or energy failure may result in either apoptosis or necrosis.

Calcium accumulation in cells, a known activator of some apoptotic pathways, can result from the uncontrolled release of excitotoxic neurotransmitters which is often seen during ischemia (Kristián and Siesjö, 1998). Alternatively, the overproduction of oxidants during ischemia can also lead to intracellular damage to DNA and other cellular components. Free radical stress stimulates the activation of many cellular death cascades (Chan, 1996). Regardless of the stimulus, the final common executioner of *most* cellular death pathways during apoptosis is the activation of caspases.

Caspase-3 is synthesized as an inactive 32kDa proenzyme that is proteolytically activated by an unknown mechanism. *In vitro* evidence suggests that caspase-3 may be activated by cleavage of itself (autoprocessing) or by other caspases (Nicholson, 1996; Nicholson *et al.*, 1995; Fernandes-Alnemri *et al.*, 1995). Cleavage results in the formation of 17kDa, or 20kDa subunits depending on the signal (Erhardt and Cooper, 1996), and 12kDa subunits which heterodimerize and form a tetramer (Nicholson *et al.*, 1995). Human caspase-3 is more closely related to ced-3 of *c. elegans* than other members of the caspase family. Based on the nucleotide sequence

caspase-3 is seen to have a 35% homology (58% similarity) to ced-3 (Fernandes-Alnemri *et al.*, 1995).

Developmental programmed cell death (PCD) of interdigital hindlimbs of chick embryos (Milligan *et al.*, 95) and cultured mice forepaw explants (Jacobson, 1996) can be prevented with inhibitors of Interleukin-1β converting enzyme (ICE, caspase-1) such as Ac-YVAD-cmk. Despite this contribution, ICE appears to be dispensable for developmental PCD in mice (Kuida *et al.*, 1995) suggesting a redundant role of this protease in developmental PCD. Nevertheless, transgenic caspase-1 gene knockout mice sustain less damage than wild type mice following cerebral ischemic insult (Friedlander *et al.*, 1997). Additional *in vivo* studies with inhibitors of ICE and ICE-like caspases suggest a decreased infarct volume in mice (Hara *et al.*, 1997) and rats (Loddick and Rothwell, 1997). The use of the broad-spectrum caspase inhibitor z-VAD-DCB appears to be effective in attenuating ischemic brain damage (Loddick and Rothwell, 1997). However, the role of ICE inhibition in preventing apoptosis still remains inconclusive. ICE expression appears to remain constant even up to 16-24hr after reperfusion following MCA occulsion despite expansion of the infarct area (Asahi *et al.*, 1997). This suggests that ICE may not be a major initiator of delayed neuronal death, but ICE inhibition may instead decrease neuronal damage by preventing an inflammatory response and tissue necrosis.

Inhibition of caspase-3-like proteases provide protection in rodent brains subjected to focal ischemia, direct excitotoxic insults (Hara *et al.*, 1997) or traumatic brain injury (Yakovlev, 1998). In contrast to ICE, the role of caspase-3 is thought to

be essential in neuronal programmed cell death during development. Targeted gene disruption of caspase-3 results in a lethal mutation in mice suggesting a unique non-redundant role of this protease in developmental PCD (Kuida *et al.*, 1996). Namura *et al.* (1998) have recently shown an increase in the cleavage of pro-caspase-3 to its active form following cerebral ischemia. In another recent study, the caspase-3 like protease inhibitor z-DEVD-fmk injected intracerebroventricularly (ICV) reduced the delayed neuronal death after a mild focal ischemic insult (Endres *et al.*, 1998). However, the neuroprotective effects of this inhibitor given systemically were not tested.

Transient global ischemia in adult rats, a model that simulates circulatory arrest in humans, results in delayed neuronal death of certain brain regions which have been suggested to involve an apoptotic type of cell death. In these studies, the hippocampus and caudate putamen have been shown to have increased caspase-3-like gene expression (Asahi *et al.*, 1997; Ni *et al.*, 1998) and increased caspase-3 protease activity (Chen, 1998). Following permanent middle cerebral artery occlusion, caspase-3 gene expression was seen to be upregulated in rats as early as one hour and at 24hr after reperfusion. Although there was an absence of punctate chromatin condensation, a morphological characteristic of apoptosis (Asahi *et al.*, 1997), at late time points there was a characteristic apoptotic ladder appearance of the DNA on agarose gels. This suggests that the delayed neuronal death may have an apoptotic component (Asahi *et al.*, 1997).

Studies examining the role of caspases during cell death in neonatal hypoxia-ischemia are limited. One recent study has examined the effect of caspase inhibition

using a 7 day old model of hypoxia-ischemia and a broad spectrum caspase inhibitor – boc-aspartyl(OMe)-fluoromethylketone (BAF). Both systemic and intracerebroventricular injections of this drug provided protection from cerebral hypoxic-ischemic injury (Cheng *et al.*, 1998). Whether a specific inhibition of caspase-3 also provides protection is not known.

Age dependence of ischemic changes in brain

One model of hypoxia-ischemia that allows the study of ischemic mechanisms at different ages is a rat model of unilateral carotid artery occlusion followed by hypoxia. This model can be performed using rats one to four weeks of age where different durations of hypoxia are required to produce similar severities of damage (Tuor *et al.*, 1996). This is related to the fact that the rat brain matures substantially between one and four weeks of age and ontogenic changes include a decrease in water content, an increase in myelin content and an increase in aerobic glycolosis (Tuor *et al.*, 1996). Furthermore, the responses observed following an ischemic insult in 4 week old animals may not resemble those of 1 week old animals (Tuor *et al.*, 1995).

Hypothesis

Cerebral hypoxia-ischemia produces severe alterations of the cellular homeostatic mechanisms resulting in cell death with a greater apoptotic component in developing brain than older brain. This is reflected by a difference in the induction and activation of caspase-3 during ischemia induced in immature and older brain.

Purpose of the present studies

The mode of cell death which occurs during cerebral hypoxic-ischemic injury in mature and immature brain is not fully understood. Evidence suggests that a portion of the cellular death mechanism is genetically controlled.

The objectives of the present studies were as follows:

1. To provide morphological evidence for apoptotic and necrotic cell death in mature and immature animals using light microscopy.
2. To determine whether there is a potential role for caspase-3 activation in cell death following ischemia in neonatal and older rats.
3. To test the efficacy of a specific caspase-3 specific inhibitor as a neuroprotective agent when administered systemically to 7 day old rats.

Methods

Selection of Animals for studies

Pregnant rats (Wistar) were obtained from Charles River laboratories and gave birth approximately 7 days following arrival. Following birth pups were chosen at random. Animals were assigned to the appropriate age groups - 1, 2, or 4 weeks of age depending on the experiment. Animals assigned to the 4-week old age group were weaned at 3 weeks of age.

Hypoxia-ischemia model

All animals were treated in accordance with the guidelines provided by the Canadian Council on Animal Care and experiments were approved by the local animal care committee. Cerebral hypoxia-ischemia was produced as previously described (Tuor *et al.*, 1998). The cervical incision site was infiltrated with 2% lidocaine and 0.1ml/10g saline was injected intraperitonially to compensate for fluid losses. Anaesthesia was induced with 3-4% isoflurane and then maintained with 1.5-2.5% isoflurane during the isolation and ligation of the right carotid artery. In the sham control group, the carotid artery was isolated but not ligated. Following surgery the rats were allowed to recover from anesthesia for 2-3 h with the dam. Rats were then subjected to hypoxia (8% 0_2 / 92% N_2) at 37^{o}C for a duration of 2h, 45min, or 30min for the 1, 2 and 4 week old animals, respectively. The reduction in hypoxia time in older animals is necessary in order to obtain a comparable area of infarction

without excessive mortality (Tuor *et al.*, 1995). Sham operated control rats were not subjected to hypoxia. Prior to euthanasia rats were injected intraperitonially with pentobarbital (80 mg/kg).

Morphology study

Animals used in the morphological study (6 per group) were perfused with 10% formalin in phosphate buffer either 24 or 48 hours after hypoxia. The brains were removed, stored in fixative, cut into coronal blocks, and then embedded in paraffin. Paraffin sections (2μm thick) were stained with hematoxylin and eosin. Brain sections were analyzed by the investigator blinded to the age of the samples. Dead or dying cells were identified using light microscopy at 400 × ocular magnification and were classified into two main groups. Cells with punctate chromatin were those which contained two or more oval bodies of condensed chromatin. Pyknotic cells contained nuclei with condensed chromatin having an irregularly shrunken or scalloped appearance (Garcia, 1993). Cells which contained a condensed nucleus with a single rounded body of chromatin were rare (<5% of pyknotic cells) and were considered to be associated with the stage of chromatin condensation which precedes cytoplasmic blebbing and the formation of apoptotic bodies during apoptosis. These cells were not included in either classification. Layer 2-5 of parietal cortex was chosen as the region of interest for quantitative analysis because the severity of damage was similar in this region for all ages. For each animal, cells were counted in standardized 66μm^2 areas of four adjacent fields from three different sections with the aid of image analysis (MCID, Imaging Research, St.

Catherines, ON). The contralateral cortex in the same sections served as a control. Data are reported as means ± SEM. Differences between means were compared using a one-way analysis of variance (ANOVA) and Tukey's test.

Detection of caspase-3 by enzyme activity and western blotting

Animal Preparation

Animals used to investigate caspase-3 activity in the brain were decapitated and the head flash frozen in liquid N_2 or isopentane cooled with dry ice (<-65°C). Three animals per group were sacrificed either 4 or 18 hours post-hypoxia-ischemia at 1 and 4 wks of age. Sham operated controls were decapitated and the heads frozen 2-3 hours following sham surgery for both the 1 and 4wk age groups. Frozen tissue was stored at -80°C until extracts were prepared.

Preparation of cytosol extracts for caspase activity

The left and right hemispheres of cortical tissue were dissected from frozen brain and thawed in lysis buffer containing 10mM Hepes/KOH (pH 7.2), 2mM EDTA, 0.1% CHAPS, 5mM dithiothreitol, 1mM phenylmethylsulphonylfluoride, 10 μg/ml pepstatin A, 20μg/ml leupeptin, and 10μg/ml aprotinin (Nicholson, 1995). Tissue was homogenized using 20 to 30 strokes in a glass homogenizer and kept on ice for 10 minutes. Homogenates were centrifuged at 13,000 × *g* for 30 minutes at 4°C (Yakovlev, 1997). Supernatants were aliquoted and transferred to new tubes and

stored at –80°C until used. Protein concentrations were determined using a modified BCA protein assay (*Pierce*) including an acetone precipitation step to eliminate interfering substances.

Preparation of total cell lysates for Western blotting of caspase-3

Tissue from frozen brain tissue was homogenized in lysis buffer (50mM glycerol; 25mM Tris HCl; pH 8, 10mM EDTA; 1mM PMSF; 10μM leupeptin; 1μM pepstatin A; 0.5μg/ml aprotinin) and incubated on ice for 30 min. The extracts were centrifuged for 30min at 12,000 × *g*. The supernatant was removed and centrifuged again at 12,000 × *g* for 30min. This supernatant, which contained the whole cell lysate, was aliquoted and frozen at -80°C until used. Protein concentrations were determined by the BCA protein assay (*Pierce*).

Assay for caspase activity

Prior to beginning enzyme activity analysis of samples, a standard curve was prepared with known amounts of *7-amino-4-methyl-coumarin* (AMC, *Sigma*). Caspase activity assays were conducted using 5-20μg of cytosolic protein with 40μM Ac-DEVD-AMC (*BIOMOL*) at room temperature following preincubation of enzymes at 35°C for 30 min. The time course of AMC release was followed using a spectrofluorometer (Spex, Fluoromax) over 30 minutes at an excitation wavelength of 380nm and an emission wavelength of 460 nm. Linear regression analysis was performed to obtain the rate of reaction. Activity is expressed as nmol of AMC released/minute/mg of protein.

To confirm AMC release was due to caspase-3 activity an additional sample was repeated with varying amounts of the reversible caspase-3 inhibitor Ac-DEVD-CHO (*BIOMOL*).

Western blot analysis of caspase-3

Cell lysates prepared above were lyophilized with 20µl of 2× SDS PAGE sample buffer (1× buffer = 62.5 mM Tris-HCl pH 6.8; 1% SDS; 10% glycerol; 0.005% bromophenol blue; 5% β-mercaptoethanol) at 75µg of protein. For detection of caspase-3 protein, samples were electrophoretically resolved using 20% SDS-polyacrylamide gels (mini-PROTEAN, *BioRad*) at a constant current of 10mAmps. Proteins were then transferred at 4°C and 75volts for 90min to polyvinyldiflouride membrane (PVDF, *Millipore* or *BioRad*) in transfer buffer (25mM Tris-HCl pH 7.5, 190mM glycine, 20% methanol).

Membranes were incubated for 90 min at room temperature in blocking solution PBS-TM (10% Milk in 10mM Tris-HCl pH 7.5; 0.1 M NaCl; 1 mM EDTA; 0.1% Tween 20). Polyclonal anti-CPP32/caspase-3 antibody (generous gift from J. Goldstein; diluted 1:1000; Wang *et al.*, 1996) was added in fresh blocking solution and incubated for 90 min at room temperature. Secondary (HRP-conjugated anti-rabbit) antibody (1:10000 dilution) was applied in PBS-T after 3 washes of 5 min in PBS-T. Following a 60 min incubation, the membranes were washed 3 times with PBS-T (0.3% Tween-20) and 3 times with PBS-T (0.1% Tween-20) and the HRP detected using an ECL chemiluminescent system (*Pierce*, Supersignal).

Effects of z-DEVD.fmk treatment on hypoxic-ischemic damage

The neuroprotective effects of caspase-3 specific inhibitor were tested *in vivo*. Animals were injected with either vehicle (0.03 or 0.06% dimethylsulfoxide (DMSO); n=9), or z-DEVD.fmk (*Calbiochem*) at a low dose (1.5μg/g body weight; n=6) or a higher dose (3μg/g body weight; n=6). These two doses were chosen based on previous studies in which this inhibitor was injected intracerebroventricularly (IVC) where the doses were converted assuming a total body instead of brain volume of distribution (Hara *et al.*, 1997; Loddick *et al.*, 1996; Yakovlev *et al.*, 1997). Intraperitonial injections of half the total dose were made 30 min pre-hypoxia and immediately post-hypoxia. An additional four animals were injected with 6μg/g body weight total pre- and post-hypoxia. Following hypoxia, blood glucose levels were measured from a drop of blood from the tail vein in all animals. Body weights were measured daily beginning the day of the surgery and until the day sacrificed. Following 72 hours of recovery from hypoxia, animals were decapitated and the brains were fixed in formalin. The brain was cut into coronal blocks and then embedded in paraffin. Paraffin sections (8μm thick) were stained with hematoxylin and eosin. Infarct areas were measured using an image analysis system (MCID). The area of infarcted hemisphere was calculated by subtracting the area of the undamaged tissue in ipsalateral hemisphere from the area of the contralateral hemisphere.

Statistical analysis

Data are presented as mean ± SEM. Differences between means of the morphological classifications were made using a one-way analysis of variance

(ANOVA) and Tukey's test. Statistical comparisons of the caspase assay data and blood glucose measurements were made using multiple comparison ANOVA followed by Dunnett's test. $P<0.05$ was considered statistically significant. Infarct volumes were analysed using a 2 (brain section) by 4 (dose) factorial design ANOVA.

Results

Morphology study

The morphological characteristics exhibited by individual cells were categorized as either pyknotic or punctate chromatin condensation in 1, 2 and 4wk old animals. Ipsilateral to the carotid occlusion, punctate chromatin condensation was frequently observed in cortical sections of rats at 1 week of age (82.7% of total cells). The frequency progressively decreased in 2 week (18.1%) and 4 week (7.1%) old rats while the number of pyknotic and/or eosinophilic cells progressively increased (Figures 1 and 2). On the side contralateral to the carotid ligation there were no pyknotic cells in any of the rats. Zero to five cells with punctate chromatin were identified in the entire contralateral cortex of 1 week old rats and no such cells were observed in 2 and 4 week old animals. Several 2 week old rats (3/6) showed neurons with fragmented chromatin and eosinophilic cytoplasm in the hippocampal CA-3 region ipsilateral to the ligated carotid artery.

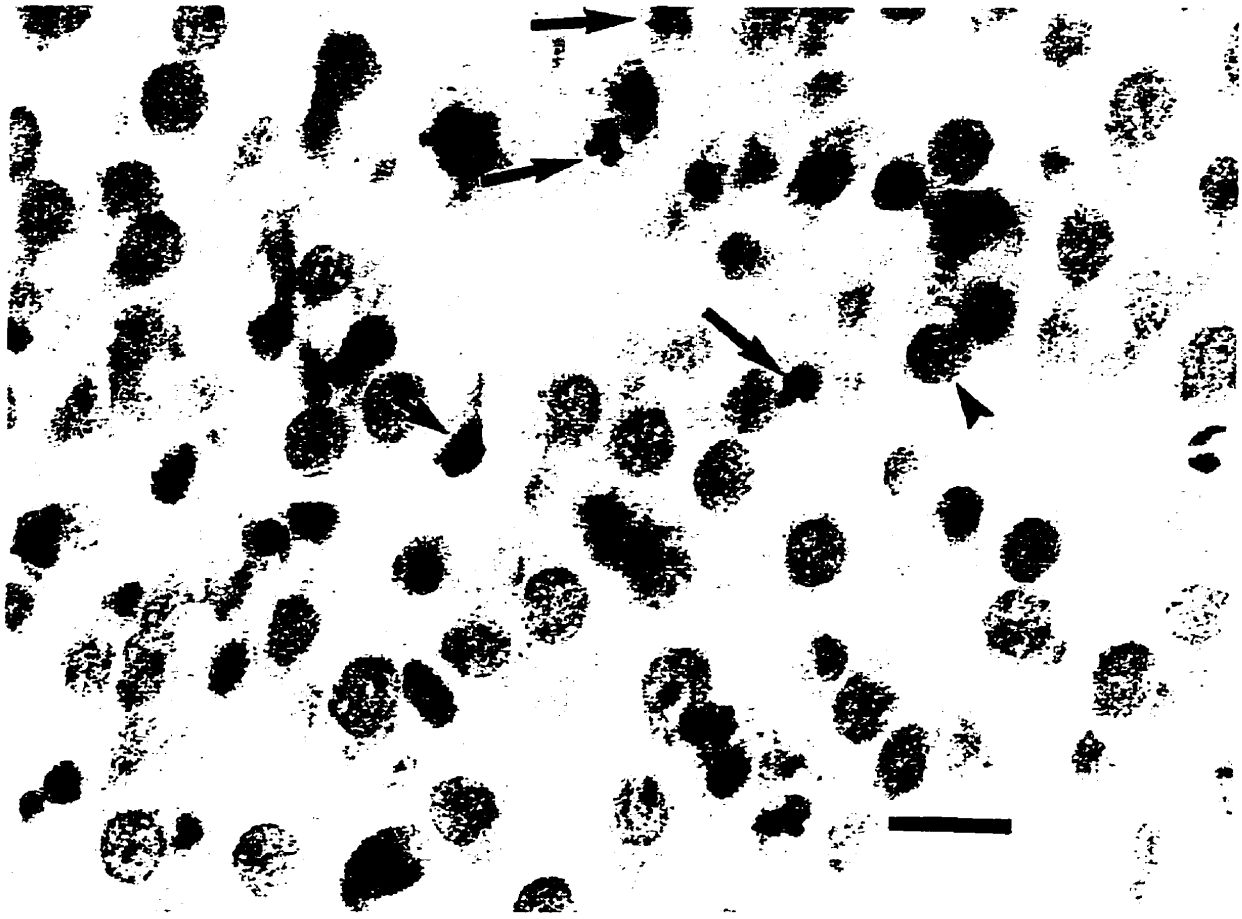

Figure 1 Hematoxylin and eosin (H and E) staining of cerebral cortex from 1 week old rat showing nuclei from an intact neuron (arrowhead), punctate chromatin condensation (large arrows), and necrotic pyknosis (small arrow). Bar = 10μm.

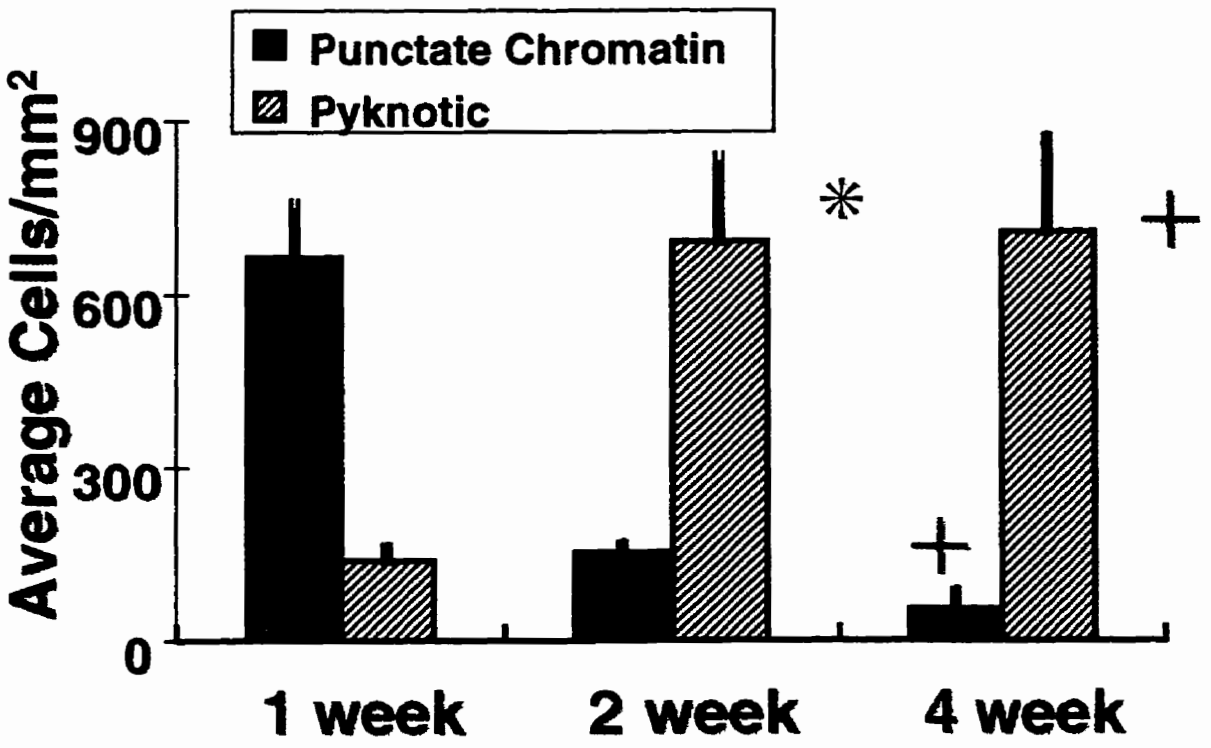

Figure 2 Bar graph showing quantitation of pyknotic nuclei and punctate chromatin condensation at various ages. *P<0.007, different from 1 week; +P<0.05, different from 1 week.

Detection of caspase-3-like activity in ischemic brain tissue

Preliminary experiments were performed using Ac-DEVD-*p*-nitroanilide. This colorimetric substrate was relatively insensitive in our system and its use was abandoned. The fluorogenic substrate Ac-DEVD-AMC was more sensitive and was used to measure caspase-3-like activity in control and ischemic brain tissue lysates. This fluorometric assay detects the shift in fluorescence emission of AMC after cleavage from DEVD-AMC as measured in a fluorometer at 460nm (Gurta, *et al.*, 1997). Cleavage was measured over 30 minutes and compared to a standard curve prepared with known quantities of 7-amino-4-methyl coumarin (Figure 3). The rate of enzyme activity was determined by taking the linear regression analysis of the slope of the line over 30min. The values were expressed as nmol of AMC released/mg protein/min. Enzyme activity was abolished when tissue lysates were preincubated with 10 or 1μM of the irreversible caspase-3-like inhibitor Ac-DEVD-CHO (Figure 4). The inhibitor alone did not affect the standard curve.

Caspase-3 like activity was significantly increased in 1 and 4week old animals exposed to a hypoxic-ischemic insult when compared with sham operated control animals (Figure 5). In 1wk old animals activity increased progressively from 4 to 18 hrs. Four week old animals also showed an increase in activity at 4 and 18 hours after ischemia. When one week and 4wk old animals were compared at equivalent time points, activity was significantly greater in the 1wk old animals when compared to 4wk old animals.

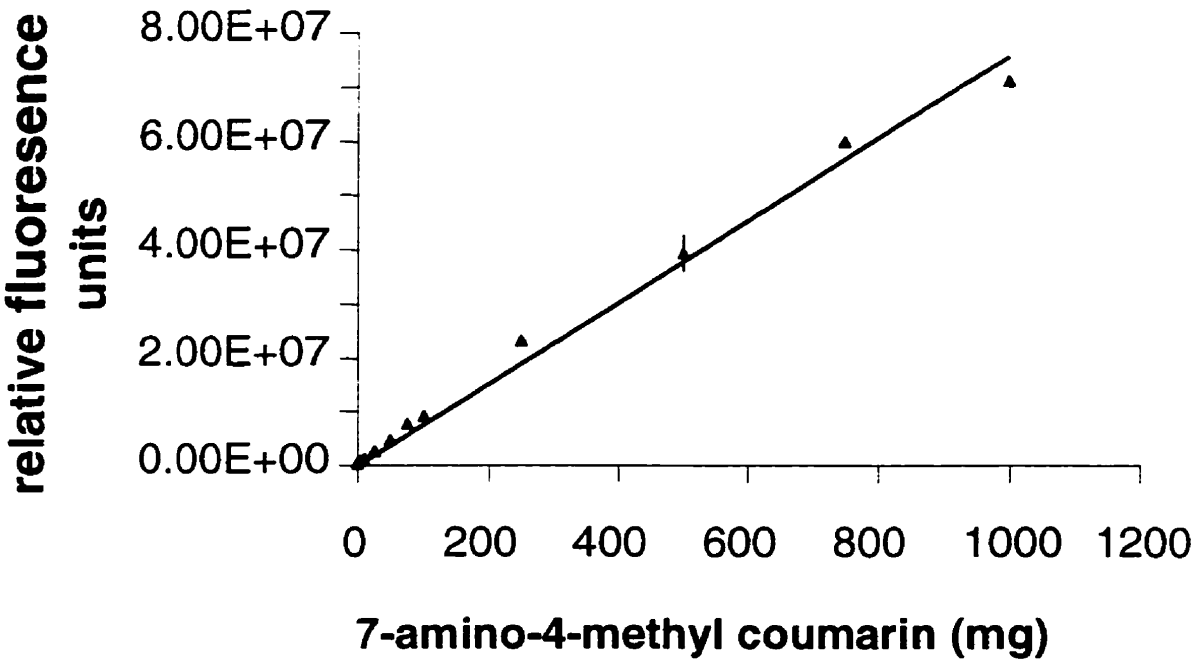

Figure 3 Standard curve prepared from known amounts of 7-amino-4-methyl-coumarin.

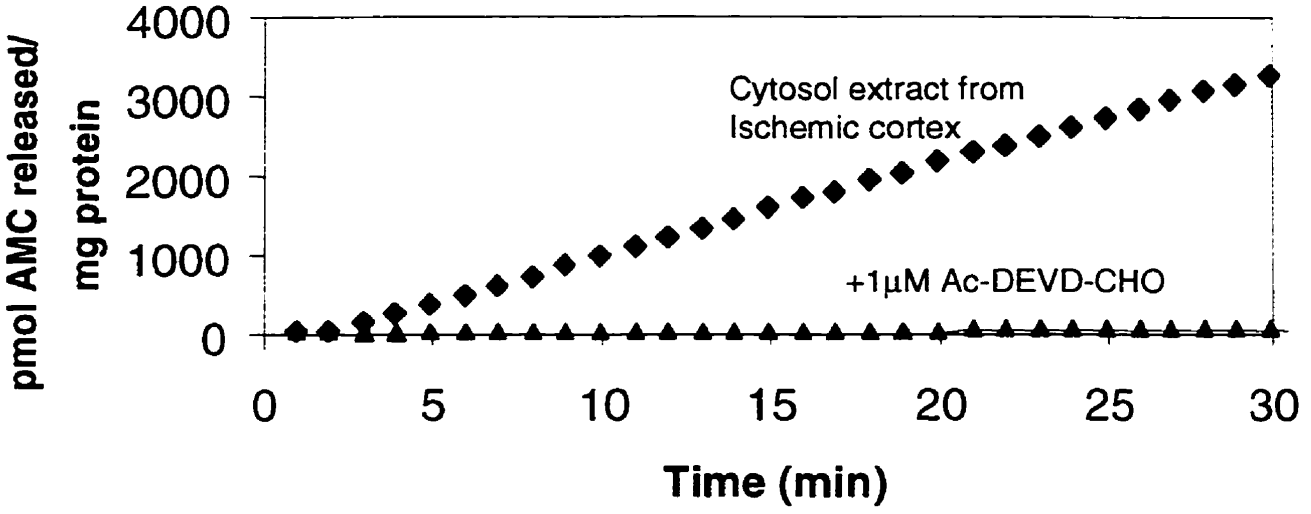

Figure 4 Cleavage of the caspase-3 fluorogenic substrate Ac-DEVD-AMC in homogenates from ischemic brain tissue. Reaction was monitored every minute for 30 min by spectrofluorometry. Preincubation with 1μM of the reversible caspase-3 aldehyde inhibitor Ac-DEVD-CHO completely abolished activity. Sample shown was prepared from a 1 week old animal at 18 hours post-hypoxia.

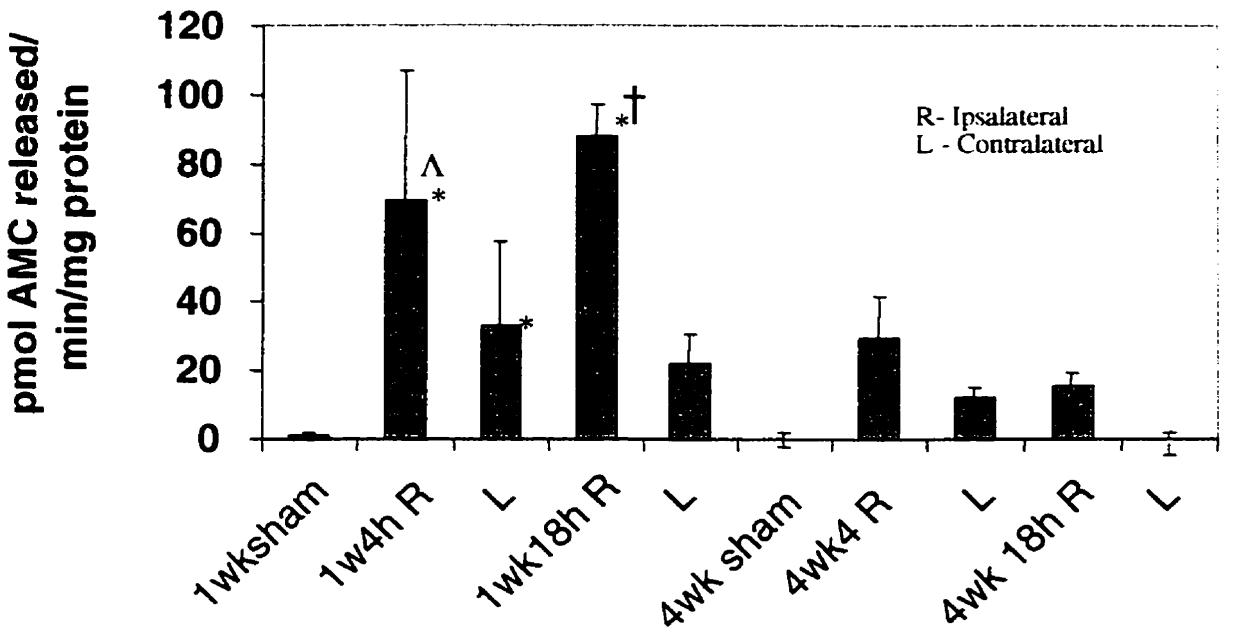

Figure 5 Caspase-3 activity measured with the fluorometric substrate Ac-DEVD-AMC in homogenates from ischemic brain tissue in 1 and 4 week old animals following either sham surgery, or 4 or 18 hours post-hypoxia. Caspase-3 activity is significantly increased 4 hours and 18 hours after hypoxia-ischemia in 1 and 4 week old animals. One week old animals have a greater caspase-3 activity than 4 week old animals at the same time points. *$p<0.05$ different from sham. †$P<0.05$ different from the same time point vs. 4 week old. Λ $P=0.08$ when compared with 4 week old 4 hour.

Expression of caspase-3 immunoreactivity with Western blotting

In order to characterize altered expression of caspase-3 protein, Western blotting was performed on *cytosolic* and *total cell* lysates using a polyclonal antibody specific for caspase-3. The antibody can detect both the inactive 32kDa proform and the active 17kDa subunit (Jacobson *et al.*, 1996; Wang *et al..*, 1996). Western blotting of *cytosolic* extracts showed no significant difference in the p32 subunit for either age group or time post-hypoxia and the p17 subunit was not detected. However, an additional band (~68kDa) differentially labeled between the 1 and 4wk old animals but was unaffected by hypoxia (Figure 6).

Western blotting of the *total cell* lysates did not show a difference in the p32 subunit. However, there was an increase in the p17 signal in the 1 wk old animals sacrificed at 18 hours (2/4 samples) and 4 hours (1/3 samples). From the 4wk old age group animals sacrificed at 18 hours (1/3 samples) showed an increase in the p17 signal. The p17 subunit was not detectable in sham animals (0/3 1wk olds and 0/3 4wk olds) and 4 wk old animals sacrificed 4 hours post-hypoxia (0/3 animals) (Figures 7 and 8). The additional band above the p32 band appeared at about 36-38kDa in the 1 and 4 week old extracts. This additional band was unaffected by hypoxia.

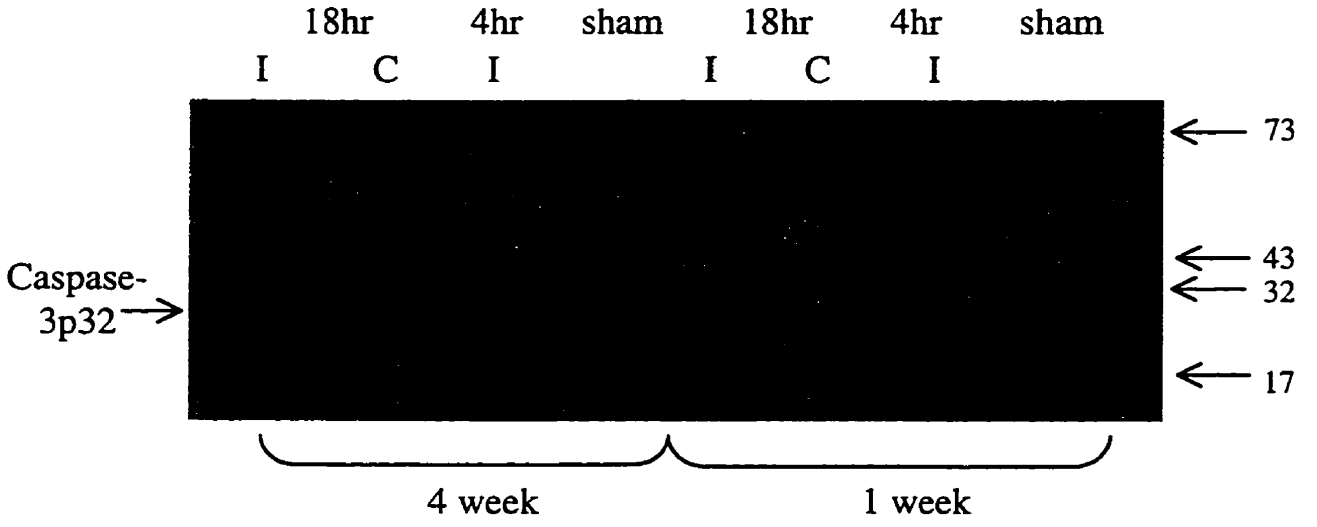

Figure 6 Western blot using a polyclonal caspase-3 specific antibody on *cytosol* extracts. Caspase-3p32 isoform in *cytosol* extracts does not appear to be affected by hypoxia. An additional band (~68kDa) appears differentially labeled between the 1wk and 4wk old animals. The p17 active subunit was not detectable. Samples are 1 week and 4 week old sham, 4-, and 18-hours post-hypoxia either (I) ipsalateral and (C) contralateral hemispheres.

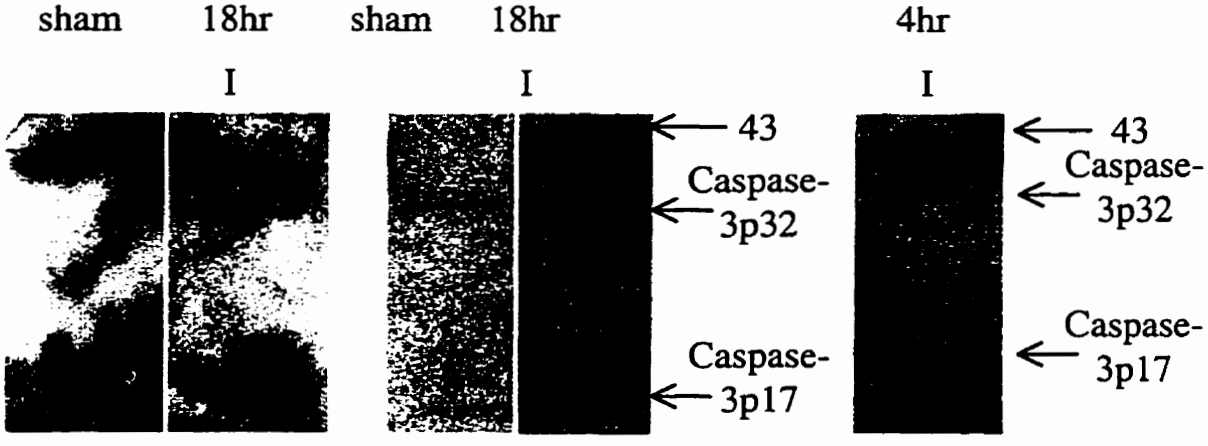

Figure 7 Western blot using a caspase-3 specific antibody of *total cell* lysates of 1 week old animals. Caspase-3p32 isoform does not appear to be affected by hypoxia. The additional 68kDa band did not appear in *total cell* lysates. The caspase-3p17 signal is detectable at 18 hours post-hypoxia (2 of 4 samples) and at 4 hours (1 of 3 samples) post-hypoxia where (I) ipsalateral

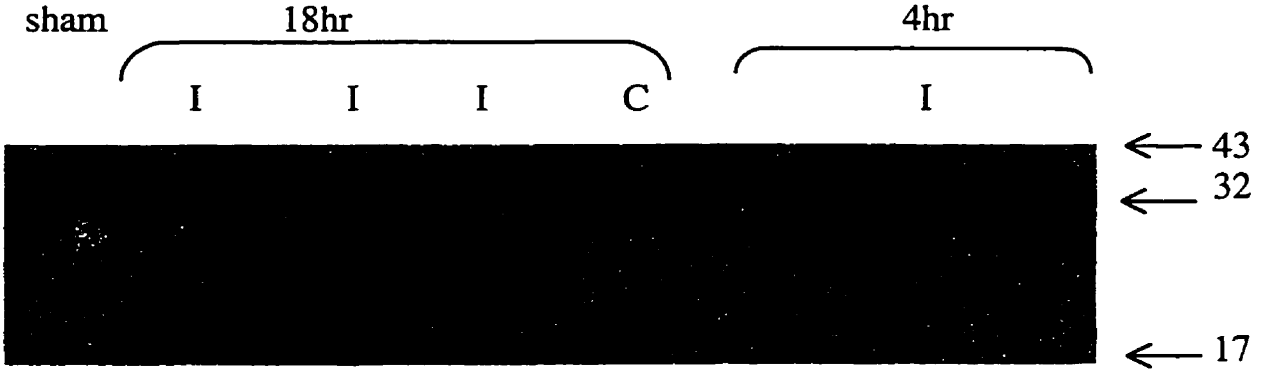

Figure 8 Western blot using a caspase-3 specific antibody on *total cell* lysates of 4 week old animals. Caspase-3p32 isoform does not appear be affected by hypoxia. Caspase-3p17 signal is detectable in samples prepared 18hours post-hypoxia but not in sham or 4 hours post-hypoxia where (I) ipsalateral and (C) contralateral hemispheres.

In vivo drug inhibitor study

Unilateral occlusion of the common carotid artery followed by exposure of animals to 8% oxygen for 2 hours reproducibly causes ischemic brain injury (Tuor *et al.*, 1998). In the present study, this well characterized model was used to test the neuroprotective effects of a membrane permeable caspase-3 specific inhibitor. Following hypoxia-ischemia animals were periodically checked for feeding and any seizure activity. All animals demonstrated normal behavior and fed well. There was no significant difference in average body weight gain between vehicle and drug treated animals (data not shown). Vehicle treated animals had lower blood glucose levels than drug treated animals (Table II).

Table II Blood glucose measurements immediately following hypoxia

Treatment	***Blood glucose***
Vehicle	1.8 ± 0.4
Low dose z-DEVD-fmk 1.5μg/g body weight	3.3 ± 0.6*
Intermediate dose z-DEVD-fmk 3μg/g body weight	3.1 ± 0.5
High dose z-DEVD-fmk 6μg/g body weight	2.0 ± 0.6

*Sig. different from vehicle $p<0.05$

Following 72 hours of recovery post-hypoxia animals were overdosed with isoflurane anesthesia and decapitated. The approximate size of the infarct was estimated visually and noted (data not shown) and was similar in the different treatment groups ranging from small to large infarcts in the hemisphere ipsilateral to

the occlusion. The area of infarction was similar to control for the different treatment groups (figure 9). However, animals administered the low dose (1.5μg/g body weight) and high dose (3μg/g body weight) z-DEVD-fmk had a slight reduction in infarct size but this was not statistically significant. The infarct size of the high dose treatment group appeared slightly greater than control but this was also not significantly different.

The morphological appearance of cells from drug and vehicle treated animals was not significantly different. Punctate chromatin condensation was detectable in all animals and this was more prevalent in regions on the border of the infarct zone. Both drug and vehicle treated animals showed punctate chromatin within the infarct zone.

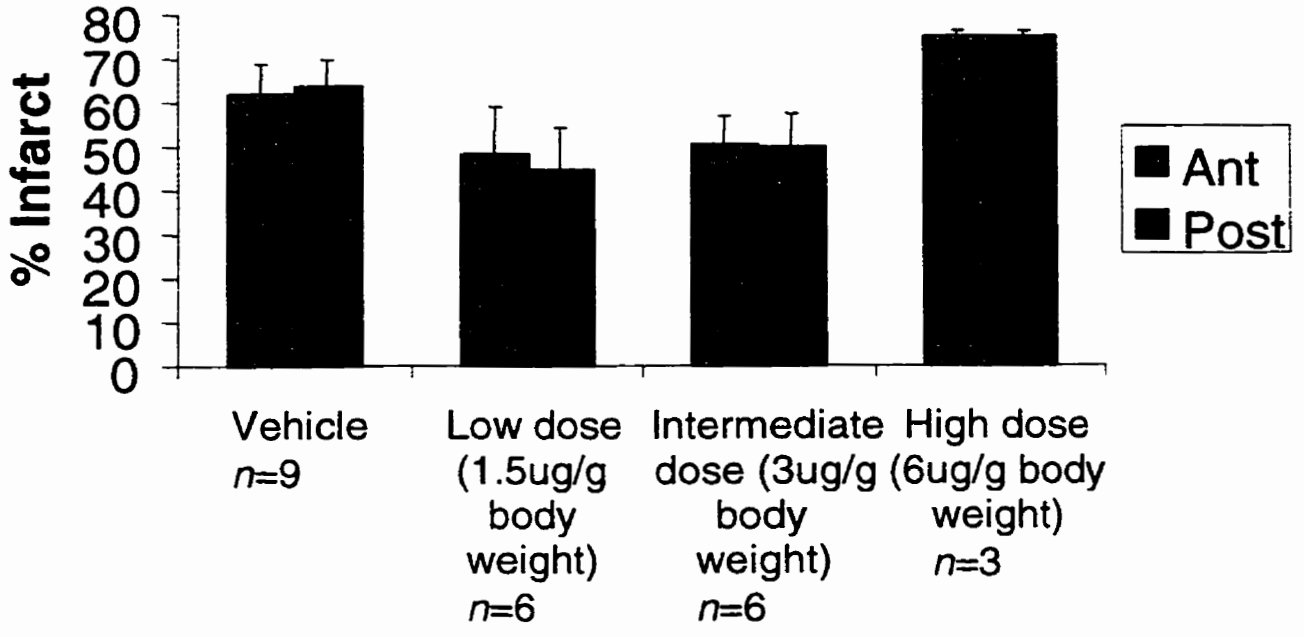

Figure 9 Infarct sizes measured following either vehicle (n=9), or low dose (1.5µg/g body weight, n=6), or higher dose (3µg/g body weight, n=6) z-DEVD-fmk. The reduction in infarct size was not significant, but low dose treatment had a P=0.08 when compared to vehicle treated. The highest dose (6µg/g body weight, n=3) appeared to have a greater infarct size.

Discussion

Morphological Study

The present studies suggest a role for apoptosis in cerebral hypoxia-ischemia in neonatal and to a lesser extent in more mature rats. Many investigators have used *in situ* labeling with terminal deoxyribonucleotidyl transferase-mediated biotin-16-dUTP nick-end labeling (TUNEL assay) to demonstrate the appearance of DNA fragments suggestive of apoptosis in both mature and immature animal models of ischemic injury (Li *et al.*, 1995a; Ferrer *et al.*, 1994). However, caution must be exercised when interpreting data attained from TUNEL labeling. Necrotic cells, with random DNA breaks, as well as apoptotic cells with free 3'-OH ends are labeled (Charriaut-Marlangue and Ben-Ari, 1995). On close examination necrotic cells may show a diffuse cytosolic staining pattern due to random DNA breaks, whereas staining within the nucleus of cells with punctate chromatin condensation is usually a characteristic of apoptotic cells allowing distinction between the two (Charriaut-Marlangue and Ben-Ari, 1995).

The present data were obtained using morphological criteria to categorize cell death as either apoptotic or necrotic, and apoptotic morphology appears to be less prevalent in more mature brain. This is consistent with previous findings by other labs who have generally failed to find an apoptotic morphology in adult models of ischemia (Deshpande *et al.*, 1993; van Lookeren-Campagne and Gill, 1996; Petito *et al, 1997*). However, notable exceptions are the penumbral areas of regions of infarct following focal ischemia (Charriault-Marlangue *et al.* 1996; Ferrer *et al.*, 1994; Li *et*

al., 1995a and b; States at al, 1996) or areas of delayed neuronal death in the hippocampus following transient global ischemia (Kirino, 1982; Kirino, 1996). The present study shows that punctate chromatin condensation can be readily observed in neonatal rats following hypoxia-ischemia in the brain. Indeed, others have seen punctate chromatin condensation with hematoxylin and eosin (H&E) staining using the infant rat model (postnatal days 3-7) in naturally occurring cell death (Ferrer *et al.*, 1994), hypoxic ischemic injury (Ferrer *et al.*, 1994) and hyperoxia induced damage (Ahdab-Barmada *et al.*, 1986). However, the present study appears to be the first to demonstrate a marked difference in the prevalence of punctate chromatin morphology depending on age.

The interpretation that this indicates a more prevalent apoptotic cell death in neonates is debateable. Some investigators have suggested a continuum between apoptosis and necrosis (de Torres *et al.*, 1997; Raffray and Cohen, 1997). Others have stressed the confusion in the categorization of apoptotic and necrotic cells in ischemic injury based on morphological appearance (Farber, 1994). For instance, the morphological appearance of apoptosis is often difficult to distinguish from necrotic cells which have ruptured (karyorrhectic), as both may be seen as punctate chromatin condensation. Furthermore, several authors have equated the two terms (Edwards *et al.*, 1995; Majno and Joris, 1995). However, in light of recent evidence using agarose gel electrophoresis of fragmented DNA (MacManus *et al.*, 1995; Hill *et al.*, 1995) and additional studies from our animals, there is reason to believe that many of the karyorrhectic cells are apoptotic bodies. To help support this we have also found

evidence for some age dependence in proteases involved in the apoptotic pathway as discussed below.

Role of Caspase-3

Western blotting

The role of caspase-3 in the execution of apoptosis in cell culture systems is well established. Much less is known of the role of caspases *in vivo*. The present study suggests that pro-caspase-3, which is constitutively expressed, becomes proteolytically activated during ischemic injury in both mature and immature rats. The active p17 subunit of caspase-3 can be detected using total cell lysates at later time points following the end of hypoxia. The p17 subunit was detected at 4 and 18 hours post-hypoxia in the 1 week old animals and at 18 hours in the 4 week old animals. Other investigators have shown an increase in the p17 signal at later time points of reperfusion in adults (Namura, 1998). Not all samples in the present study showed a p17 band possibly due to a varying proportion of severely ischemic tissue. In addition, the absence of the p17 signal in Western blots does not necessarily imply that active p17 caspase-3 does not exist at earlier time points. There has been evidence of caspase-3 mRNA upregulation soon after transient global ischemia (Kinoshita *et al.*, 1997) and Western blotting may simply not be sensitive enough to detect slight changes in protein expression. Alternatively, the mRNA upregulation may not be a true reflection of protein content as there may not be immediate translation into protein.

The signal intensities of the caspase-3 bands were not quantitatively compared between the 1 and 4 week old samples. There did not appear to be a significant difference in the p17 signal at 18 hours post-hypoxia, except that one sample appeared to have a much stronger signal in the 1 week old animals. In addition, one sample at 4 hours post-hypoxia showed a faint signal in the 1 week old samples, while none of the 4 week old samples had a detectable band.

Western blotting of cytosolic extracts consistently showed a different banding pattern at a molecular weight above the 32kDa pro-caspase-3 p32 specific band (~68kDa) in 1 and 4week old animals which was unaffected by hypoxia. This band was previously reported in cell culture to be unaffected by treatment conditions with this antibody (Jacobson *et al.*, 1996). Additional bands have also been reported with other antibodies used in different tissues (Krajewska *et al.*, 1997). These additional bands may represent a posttranslationally modified form of caspase-3 or cross reactivity with other caspases.

Caspase-3 like enzyme activity assay

Enzyme activity measured by the accumulation of the fluorogenic product 7-amino-4-methyl coumarin (AMC) from DEVD-AMC has, until recently, been presumed to be the result of caspase-3 activity. It is now understood that caspases-1, -4, and –7 can also cleave the substrate DEVD-AMC (Talanian *et al.*, 1997). Thus, enzyme activity measured in the present study is interpreted to be a result of caspase-3-*like* enzymes and not caspase-3 alone. Use of the inhibitor Ac-DEVD-CHO confirmed the specificity of the assay for caspase-3 like proteases. A low

concentration (1μM) was sufficient to completely inhibit activity without affecting the standard curve suggesting that caspase-3 like proteases are responsible for the enzyme activity and not the spontaneous degeneration of the substrate to yield fluorescent product. The levels of caspase-3 like activity at late time points in the 4wk old animals was similar to that measured recently following middle cerebral artery occlusion in adults early in reperfusion (Namura *et al.*, 1998).

A unique finding is that the extent of caspase-3 like activation appears to be about 3 fold higher in immature as opposed to older rats. The higher activity in the immature animals suggests that there is an increased amount of cell death by this apoptotic pathway in neonatal ischemia. Ischemic cell death in mature animals may be mediated not only by caspase-3 but also by other proteases. One possibility is a contribution of interleukin-1β converting enzyme (caspase-1; ICE) -like proteases. Once the inflammatory response cytokine interleukin-1β is released and converted by ICE it may contribute to cell death suggesting an increased prevalence of necrosis. The age dependent morphological characterization of cell death supports this hypothesis.

In vivo drug inhibitor study

Some evidence suggests that there may be a redundancy of function in the role of caspases during apoptosis. This is evident as ICE knockout mice develop normally (Kuida *et al.*, 1995) but sustain less injury during ischemic cell death. This suggests a nonessential role during development (Friedlander *et al.*, 1997). In contrast, caspase-3 plays a non-redundant role in developmental PCD as evidenced by a premature

lethality of caspase-3 knockout mice. Unfortunately the use of transgenic mice for studies of the role of caspase-3 in mature brain is limited as the lethal embryonic mutation precludes any study of function in the adult. Nevertheless, both caspases may be activated during ischemia as inhibitors of both provide some protection (Hara *et al.*, 1997; Namura *et al.*, 1998). The present study sought to determine if ischemic injury in 1 week old rats, with a large contribution of caspase-3, can be effectively reduced using a caspase-3 specific inhibitor administered systemically.

In the present study the protection from cerebral hypoxia-ischemia was very slight (not significant) using the caspase-3 like selective inhibitor. Previous studies which have used caspase inhibitors have shown dramatic reductions in infarct volume following intracerebroventricular injections of the caspase-3-like selective inhibitor (z-DEVD-fmk) and ICE-like inhibitors (z-VAD-DCB or z-YVAD-fmk) (Loddick *et al.*, 1997; Hara *et al.*, 1997). The dosing schedule used presently was similar to that in previous adult animal studies (Hara *et al.*, 1997). In addition, the doses used in this study are comparable to those used previously with the more general caspase inhibitor boc-aspartyl-fluoromethylketone (BAF) given as an intraperitoneal injection to neonatal rats (Cheng *et al.*, 1998). Thus, the negative results obtained using z-DEVD-fmk administered systemically are not readily explained by the dosing schedule or dosages used.

The slight increase in blood glucose observed in drug treated animals following hypoxia may have contributed to the trend in protection observed. In adult animal models elevated blood glucose levels may contribute to enhanced cell death (Siesjö *et al.*, 1996; Tuor *et al.*, 1996) but in neonates elevated glucose levels are

generally protective. Since blood glucose was somewhat elevated in z-DEVD-fmk treated animals, this may account for the slight trend for decrease in neuronal damage in these animals (Tuor *et al.*, 1996).

The lack of a marked neuroprotection observed in the present study could be due to three possible reasons. First, the drug may have entered the brain to prevent apoptosis, however, cell death in ischemia may be mediated by other caspases not inhibited by z-DEVD-fmk. In a study using neuronal cells in culture, it has been shown that caspases mediate the apoptotic component of cell death during oxygen-glucose deprivation (Gottron, 1997). However, the caspase-3-like specific inhibitor z-DEVD-fmk did not attenuate staurosporin-induced cell death as effectively as the pan-caspase inhibitors z-VAD-fmk or B-D-fmk in these cells. The authors suggested that z-DEVD-fmk was effective at low doses and became toxic at higher doses leading to necrosis.

A second possibility is that the tendency towards a greater infarct size observed in animals treated with higher doses of z-DEVD-fmk could be attributable to a switch from apoptotic death to necrotic death. Such a phenomenon has been observed in cells which have been treated with caspase inhibitors (Lemaire *et al.*, 1998). Thus, the inhibition of caspase-3 may prevent apoptosis but induce cell death by necrosis at higher doses.

Finally, in a previous study, significant protection was observed using the 236kDa lipid soluble caspase inhibitor BAF (Cheng *et al.*, 1998). Generally lipid soluble drugs below a molecular weight of 500kDa are able to cross the blood brain barrier (Pardridge, 1998). The caspase-3 specific inhibitor z-DEVD-fmk has a

molecular weight of 686kDa, thus, the size may preclude its entry into the brain. The poor efficacy suggests that the large size of the drug was unable to penetrate the blood brain barrier despite it being under developed in the 1wk old rats. Based on previous reports of effectiveness with the caspase-3 specific inhibitor (Namura *et al.*, 1998) it may be possible that either intracerebroventricular injections at the appropriate dosing schedule or intracarotid injections of z-DEVD-fmk at the time of surgery may prove effective in cerebral hypoxic-ischemic injury.

Summary

Although, the present study was unable to demonstrate a protective effect with the administration of a specific caspase-3 inhibitor, there is evidence for the role of caspase-3 in cell death due to cerebral hypoxia-ischemia in immature and older animals. Caspase-3 activity, a final executioner of apoptosis, was increased in lysates from hypoxic-ischemic tissue. In addition, there was also evidence for a cleavage of the inactive 32kDa proform to the active 17kda subunit. These findings support that caspase-3 contributes to cell death following cerebral hypoxia-ischemia *in vivo*.

The results from this study also demonstrate age differences in the potential role of apoptosis following cerebral hypoxia-ischemia. The increased apoptotic morphology and increased caspase-3 like activity provides evidence for a greater contribution of apoptosis in immature than older brain. These novel findings raise a plethora of questions in the accelerating pace of research in the area of cerebral ischemic injury.

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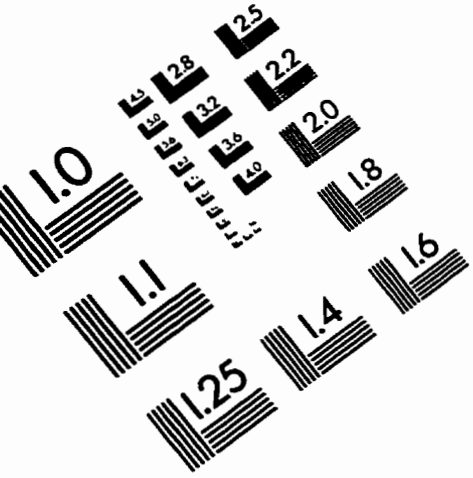
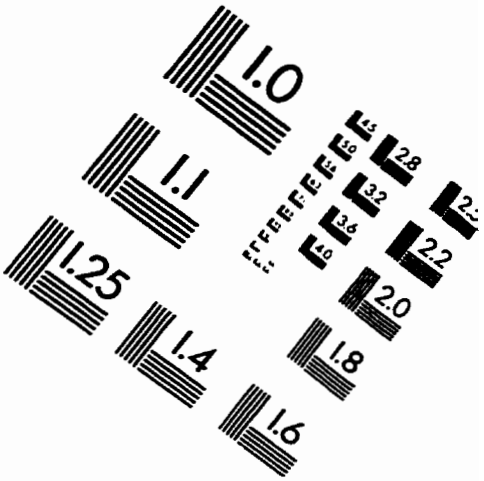
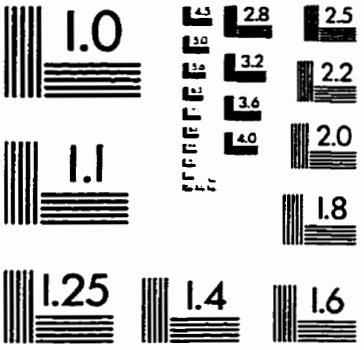
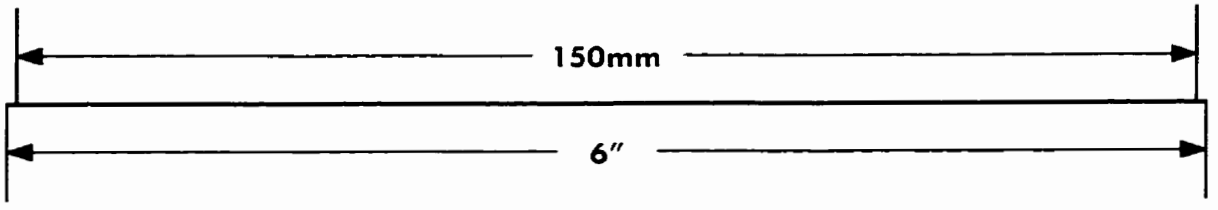
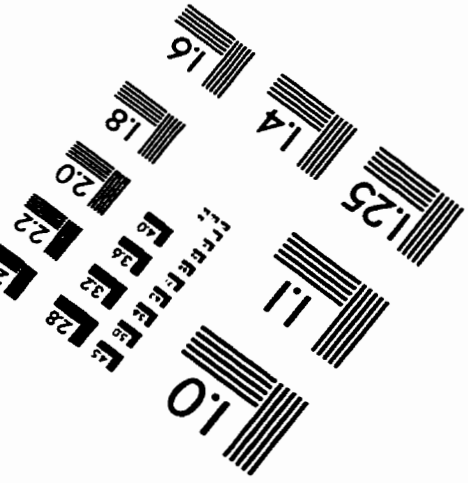
IMAGE EVALUATION TEST TARGET (QA-3)

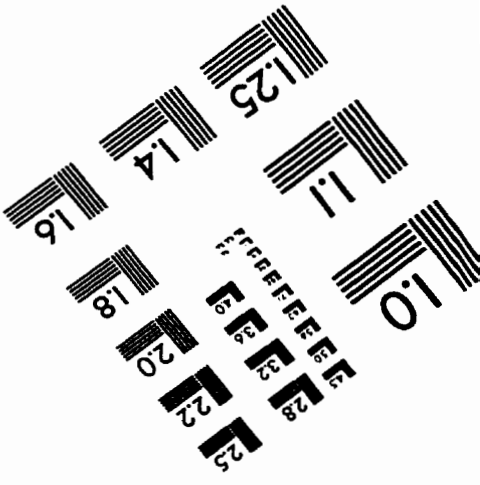












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