UNIVERSITY OF CALIFORNIA, SAN DIEGO

Microvascular Rarefaction: Capillary Stasis and Endothelial Apoptosis in a Dexamethasone-Dependent Model of Hypertension

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Bioengineering

by

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2007

The Dissertation of Edward Duc Tran is approved, and it is acceptable in quality and form for publication on microfilm:

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2007

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Acknowledgments

Thanks go to Dr. Geert W. Schmid-Schönbein, my memtor. He taught me that biomedical research should always entail the most probing and important questions. I thank him for his guidance to improvedscientific writing. I also thank him for making his enthusiasm and love for the microcirculation contagious,and for sharing the joy of watching leukocytes roll on the endothelium.

I also wish to thank Drs. Marcos Intaglietta, Paul C. Johnson, Daniel T. O'Connor, and Morton P. Printz for their suggestions and comments.

I have enjoyed working with colleagues in the Microcirculation Laboratory. I wish to thank Frank A. DeLano for the invaluable assistance in learning experimental techniques.

Finally, I would like to thank my family and friends for their unconditional love and support.

Chapter 3, in part, and chapter 4, in part, have been submitted for publication of the material as it appears in An in-vivo Analysis of Capillary Stasis and Endothelial Apoptosis in a Model of Hypertension, 2007, Tran, Edward D., Schmid-Schönbein, Geert W., Microcirculation. The dissertation author was the primary investigator and author of this paper.

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KC Mun, FA Delano, ED Tran, GW Schmid-Schonbein. "Microvascular cell death in spontaneously hypertensive rats during experimental inflammation" Microcirculation 9(5): 397-405, 2002

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ABSTRACT OF THE DISSERTATION

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Professor Geert W. Schmid-Schönbein, Chair

Recent evidence suggests that endothelial apoptosis may be a mechanism for capillary rarefaction in hypertensives. The objective of this study is to examine the early phase of cell apoptosis and capillary blood flow in single capillaries of the rat mesentery in the spontaneously hypertensive rat (SHR) and its normotensive control, the Wistar-Kyoto (WKYs) rat.

Since hypertension in the SHR is critically dependent on glucocorticoids, the animals were treated with a synthetic glucocorticoid, dexamethasone (DEX), by intraperitoneal injection and by superfusion. Capillaries with single file flow of cells in mesentery were observed in-vivo at high resolution during a period of that leads to permanent stasis without central pressure reduction. Propidium iodide (PI) was used as a marker to detect apoptosis.

Continuous observation of apoptotic cells showed that permanent stasis in capillaries is initiated by the entrapment of leukocytes at the location of an endothelial cell that had platelets attached to its membrane. The capillary endothelial cell at which such obstruction occurred may or may not be PI-positive, but after stasis became PI-positive followed by apoptosis in other endothelial cells of the same obstructed capillary. No increase in endothelial cell apoptosis was seen in capillaries without stasis, and no stasis occurred in arterioles and venules with diameters larger than capillaries and continued flow. The stasis occurred without detectable reduction in central blood pressure or arteriolar constriction. The incidence of permanent capillary stasis and total cell death in the WKY+DEX group is significantly higher than that of untreated control group, WKY, whereas there were no differences between the SHR+DEX and the SHR. Blockade of the lectin domain of L-selectin or a platelet membrane adhesion molecule (glycoprotein IIb/IIIa) blocked the development of stasis

The current in-vivo results indicate that (1) glucocorticoid, which is involved in the development of hypertension, also facilitates cell death and microvessel stasis, (2) the presence of immobilized platelets and leukocytes plays a central role in capillary stasis, and (3) capillary stasis leads to progression of endothelial apoptosis along the entire length of the capillary.

1 Introduction

1.1 Hypertension

Hypertension is probably the most important public health problem in developed countries. It is very common, asymptomatic, readily detectable, and if left untreated, often leads to lethal complications.

1.1.1 Definition

Since there is no dividing line between normal and high blood pressure, arbitrary levels have been established (by the Joint Committee on Detection, Evaluation, and Treatment of High Blood Pressure) to define persons who have an increased risk of developing a morbid cardiovascular event and will benefit from medical therapy. The blood pressure should be measured at least twice during two separate examinations after the initial screening. In adults, a diastolic pressure equal or above 90 mmHg or a systolic pressure equal or above 140 indicate hypertension.

The prevalence of hypertension depends on both the racial composition of population studied and the criteria used to define the condition. Prevalence is as high as 35%. In 90 to 95% of cases the etiology is unknown (16). Patients with arterial hypertension and no definable cause are said to have primary, essential, or idiopathic hypertension. In only a small minority of patients with elevated arterial pressure can a specific cause be identified is called secondary hypertension.

The current treatments include nondrug therapeutic intervention and drug therapy. Nondrug therapeutic intervention include: relief of stress, dietary

management, regular aerobic exercise, weight reduction, and control of other risk factors contributing to the development of arteriosclerosis. Drug therapy for hypertension is the most common reason in the US for office visits to physicians and for use of prescription drugs. In general, there are six classes of drugs: diuretics, antiadrenergic agents, vasodilators, calcium entry blockers, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin receptor antagonists.

1.1.2 Complication

Patients with hypertension die prematurely; the most common cause of death is heart disease, with stroke and renal failure also frequent, particularly in patients with significant retinopathy. Neurologic effects of long-standing hypertension often lead to retinal and central nervous system changes such as central nervous system dysfunction, cerebral infarction, cerebral hemorrhage, hypertensive encephalopathy, etc.

Chronic hypertension has enhanced microvascular complications and frank organ injury, often leading to lethal complications (40). The microvascular, cell and molecular mechanisms responsible for these complications are, however, not well defined.

One hypothesis that has been advanced as a mechanism for the enhanced arteriolar resistance in hypertensives is a decrease in the number of terminal arterioles and capillaries (21,53). This dissertation is to present an analysis of cellular mechanisms responsible for microvascular rarefaction in animal models of hypertension

1.2 Animal Models of Arterial Hypertension

Animal models of hypertension were extensively used for investigating the pathophysiology of the disease, end organ damage, and the effect of treatments on the course and complications of hypertension.

Different models showed a gradual evolution from acute (surgical approaches), to genetic (spontaneously hypertensive animals selected by cross breeding), to transgenic models. Although none of these models develop forms of arterial hypertension identical to those of human pathology, they have contributed extraordinarily to the progress of knowledge on hypertension pathophysiology, and therapeutics

1.2.1 Selected Animal Models

A Goldblatt Model of Hypertension

Harry Goldblatt first published the results of experiments of dogs in which high blood pressure was induced by constricting one or both renal arteries with an adjustable clamp. There were three different models: “two kidneys, one clamp” model; “two kidneys, two clamps” model; and “one kidney, one clamp” model. The constriction of just one main renal artery elevated blood pressure. However, the blood pressure returns gradually to the original level unless the controlateral main renal artery is also constricted or the controlateral kidney is removed. In rats, rabbits, or human, constriction of one main renal artery and leaving the other kidney intact produced sustained hypertension (14).

B Hormonal Models of Hypertension

The administration of deoxycorticosterone acetate (DOCA) in combination with a salt-diet and unilateral nephrectomy, induced a low rennin hypertension and therefore may represent a different animal model of hypertension.

C Dahl Salt-Sensitive Rats

An alternative animal model of hypertension is represented by the Dahl salt-sensitive rat in which hypertension could be induced with a high (8%) sodium diet. This rats was bred in the 1950 when Meneely and coworkers, observed ‘‘a marked degree of individual variation’’ in the blood pressure response to salt ingestion. The Dahl salt-sensitive rat has a moderately increased blood pressure with a normal salt diet, but upon salt feeding blood pressure rises steeply, to levels slightly higher than those found in spontaneously hypertensive rat (SHR). Although cardiac hypertrophy is comparable to that found in SHR (34), cardiac failure has been noted already at 4–5 months of age. Also, renal changes seem more severe than in SHR, with the development of an early proteinuria. The advantage of this model compared to SHR is that hypertension could be induced upon demand and compared to two controls: the salt-resistant strain given the high salt diet (SRS) and the salt-sensitive strain not given the high sodium diet (SSN).

D Transgenic and Knockout Models of Hypertension

Transgenic techniques represent powerful tools for the study of gene-related mechanisms of diseases such as hypertension, resulting from a complex interaction between genetic and environmental factors. The generation of the hypertensive transgenic rat line TGR(mREN2)27 bearing the murine Ren-2 gene cloned from the

DBA/2J mouse strain provided a monogenic model of hypertension. These rats develop severe hypertension, reaching 200 mmHg or higher values at 8 weeks of age in the heterozygous animals and develop pathological alterations typical of systemic hypertension. In this model high expression of the transgene in a number of extrarenal tissues is associated with increased local formation of angiotensin II. The heart develops necrosis and fibrosis, severe adrenergic dysfunction, and abnormal Ca^{2+} homeostasis. In a double transgenic rat (dTGR) the same heart alteration were found, whereas the kidneys undergo a hemolytic-uremic syndrome with vasculopathy. For this animal model, rats transgenic for the human angiotensinogen and rennin genes are crossed. These rats develop moderately severe hypertension but die due to end-organ cardiac and renal damage.

Due to the advances in mouse molecular genetics and physiology, the mouse became the animal model of choice for studying the genetic basis of hypertension (16, 17). In the last years transgenic mice carrying either the human angiotensinogen gene or the human rennin gene were developed and characterized. Because of the established species-specific interaction between angiotensinogen and renin, the blood pressure of human angiotensinogen and human rennin single transgenic mice does not differ from wild type controls. Offspring derived by crossing the two strains develop severe hypertension. This double transgenic mouse show increased plasma angiotensin II, and the kidneys develop typical renal hypertensive lesions (16,17). Tsukuba hypertensive mouse (THM), that carries human genes for both rennin and angiotensinogen represents another transgenic model of hypertension. Tsukuba

hypertensive mouse develop blood pressure levels significantly higher than control mice, cardiac hypertrophy, and renal glomerular sclerosis.

A number of renin-angiotensin system knockout mice strains have been also established. Although the loss of a single angiotensin gene has only a moderate effect on blood pressure, homozygous mice are severely hypotensive and exhibit renal abnormalities.

Mice lacking all three type of nitric oxide synthase (NOS) [neuronal (nNOS), inducible (iNOS), and endothelial (eNOS)] were developed and analyzed. Mice lacking eNOS are hypertensive and exhibit a small, but significant reduction in heart rate. Mice lacking eNOS display an abnormal developmental regulation and remodeling of arteries in response to changes in blood dynamics. Mice lacking bradykinin B2 receptor are normotensive when fed with a normal saline diet, but develop salt-sensitive hypertension after prolonged salt (3.15% NaCl) loading. Knockout mice for dopamine receptor D_{1A}, develop hypertension while maintaining normal serum electrolyte and Ca^{2+} concentrations. Mice completely lacking of atrial natriuretic peptide show a slight increase of blood pressure and develop severe hypertension after a 2 week regimen on intermediate-high salt diet (2%).

Another animal model demonstrates that the heredity is an extremely important factor in hypertension is the spontaneously hypertensive rats

1.2.2 Spontaneously Hypertensive Rats and Glucocorticoids

A The Spontaneously Hypertensive Rats

Spontaneously hypertensive rats (SHR) and stroke-prone-SHR (SHRSP) are two widely used animal models of hypertension. The animals are normotensive at birth and gradually develop severe hypertension in the first 2–4 months of life. At 6 months they show a sustained hypertension, and also exhibit a variety of organ dysfunctions. The development of the SHR strain began in 1950s by Dr. Okamoto. The selection of hypertensive rats was started by mating between Wistar Kyoto (WKY) rats with relatively high blood pressure. Through their efforts to select hypertensive offspring by repeatedly checking the blood pressure, they finally were able to establish a colony of rats developing hypertension without exception, and in 1963 reported on them as SHR.

To develop a suitable model of hypertension related to human cardiovascular pathology, a new substrain of SHR through selective inbreeding of the SHR offspring of animals that died of stroke was established. In this strain, SHRSP over 80% of the population develop stroke. Stroke-prone-SHR dies due to cerebrovascular disease and has a shorter life span. Stroke-prone-SHRs are salt sensitive in contrast to regular SHR. Spontaneously hypertensive rats generally develop hypertension with a systolic blood pressure plateau of about 200mmHg. Stroke-prone-SHR show a more rapid increase in blood pressure from a young age and reach extremely high blood pressure values of around 240 mmHg or higher at the age of 20 weeks in males and 25 weeks in females. Spontaneously hypertensive rats and SHRSP have lower body weight compared with WKY rats.

The fact that it is possible to develop a 100% hypertensive strain of rats by selective in breeding demonstrates that the heredity itself is an extremely important factor. Several studies in the last years have investigated the genes involved in the development of hypertension in SHR. Twenty four genes were found to be up-regulated and 20 were down-regulated in SHR, even if the identification of differentially expressed gene may not be an efficient method for selecting candidate genes for hypertension in the SHR–WKY system.

B Glucocorticoids

Glucocorticoids are a vital class of endogenous steroid hormones. Although named for their role in glucose homeostasis, glucocorticoids also regulate profound and diverse physiological processes including growth, development, metabolism, behavior and apoptosis, and many other functions. Produced and secreted by the adrenal cortex, levels of circulating glucocorticoids are regulated by adrenocorticotropic hormone (ACTH) largely under the control of the hypothalmic-pituitaryadrenal axis (HPA).

The actions of glucocorticoids are thought to be mediated through the glucocorticoid receptor (GR). Glucocorticoids diffuse freely across the cell membrane because of their lipophilicity. Once in the cytoplasm, they interact with the glucocorticoid receptor which mediates most, if not all, of the hormone-induced actions. The GR is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. Structure models predict both subtle and drastic changes in ligand-induced receptor conformations can direct or impede specific protein interactions responsible for downstream biological effects. Like all members

of this superfamily, the dimeric GR mediates transactivation of many target genes by binding sequence-specific recognition elements (glucocorticoid response elements, GRE) in their promoter region. The response and sensitivity to glucocorticoids varies among species, individuals, tissues, cell types, and even during the cell cycle.

Dexamethasone, which is a synthetic hormone with 25 times the glucocorticoid activity of cortisol and no mineralocorticoid activity, has often been used to examine the effects of glucocorticoids (53).

C Glucocorticoids and Hypertension

Several animal models have been useful for exploring glucocorticoid involvement in the process developing hypertension disease. The secretion of glucocorticoids by the adrenal glands is necessary for the maintenance of the high blood pressure in the spontaneously hypertensive rat, which is often studied in relation to human hypertension. Basal plasma corticosterone concentrations are significantly higher in the SHR compared with the Wistar Kyoto (WKY) rat.

The blood pressure and organ dysfunction in the SHR is dependent on glucocorticoids (53,27). Adrenalectomy in the SHR serves to normalize the blood pressure and reduce organ dysfunctions to the level of its normotensive control, the Wistar Kyoto (WKY) rat, while supplementation with a synthetic glucocorticoid, dexamethasone (DEX), at equal concentrations significantly raise the SHR blood pressure above the level of the WKY rats (29, 48). At the same time, glucocorticoids in the SHR also induce apoptosis in the thymus (45) and they raise the level of microvascular apoptosis in the SHR after they have been reduced by adrenalectomy (29).

To study the effect of glucocorticoid on hypertension we used dexamethasone-treated WKY as a model of acute hypertension.

1.3 Rarefaction

Structural rarefaction, or loss of microvessels, has been shown in human and the spontaneously hypertensive rats (SHR). Structural rarefaction has been demonstrated in several tissues of the SHR (6, 8, 18, 19, 34). More recently, a number of studies also suggest an elevated level of cell apoptosis in the thymus, brain, kidney, smooth muscle, and cardiac muscle of the SHR (10, 12, 13, 16, 28, 31, 36, 42) as well as capillary endothelial apoptosis in a Goldblatt model of hypertension (15). We therefore hypothesize that apoptosis may be a mechanism for structural rarefaction of microvessels (15, 53).

The microvessel length density in SHR skeletal muscle was reported to be 86% of the average density in WKY (53), a number that is similar to the capillary length density reduction we found here for the dexamethasone-treated Wistar rat and dexamethasone-treated WKY rat (53). The decreased microvessel length density in the dexamethasone-treated animals compared with agematched controls serves as direct evidence for structural microvascular rarefaction in this hypertensive model. The decrease in microvessel length density is contrary to the expectation that the density could increase due to skeletal muscle atrophy in the dexamethasone-treated rats.

Consistently with previous studies of rarefaction in SHR and WKY+DEX, my pilot study of the capillary networks in cremaster muscle shows the decreased

microvessel length density in the SHR, and indicated that there were fewer capillary cross-connections in the SHR (Figure 1.3)

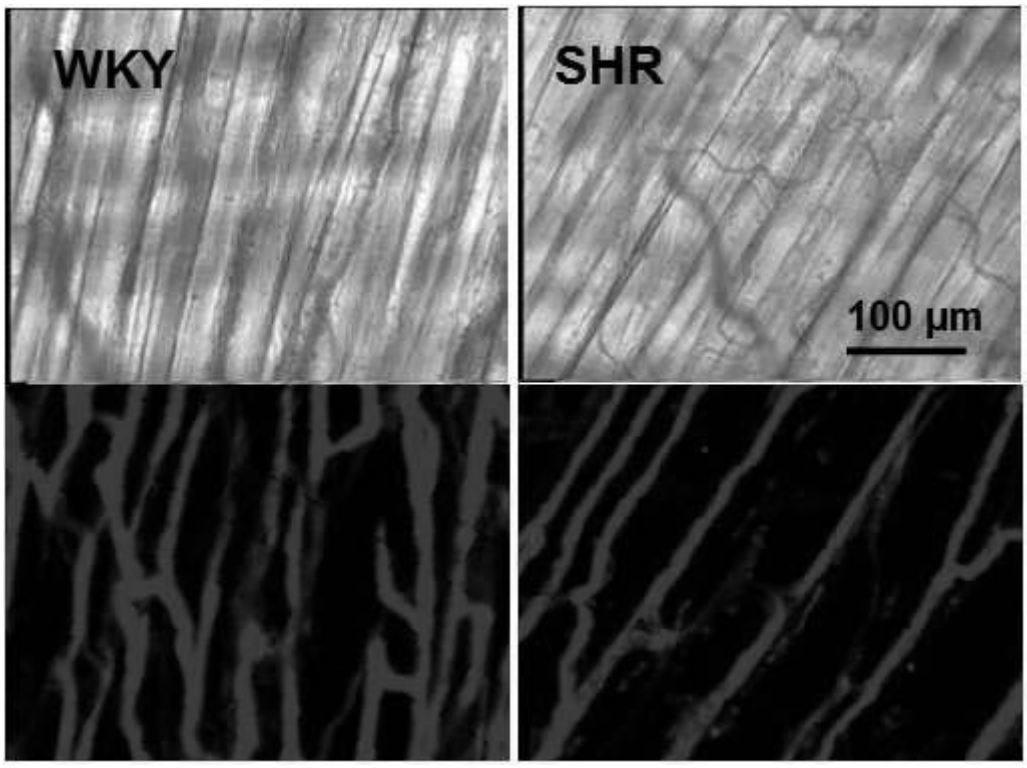

Figure 1.3 Bright field and fluorescent micrographs of microvessels stained with FITC labeled lectin in cremaster muscle of WKY and SHR.

1.4 Apoptosis

1.4.1 Overview of Cell Death

Cell death can occur by either of two distinct mechanisms, necrosis or apoptosis. In addition, certain chemical compounds and cells are said to be cytotoxic to the cell, that is, to cause its death. The two mechanisms of cell death may briefly be defined:

Necrosis ("accidental" cell death) is the pathological process which occurs when cells are exposed to a serious physical or chemical insult.

Apoptosis ("normal" or "programmed" cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes.

Apoptosis is a tightly regulated, biochemical process used by organisms for the normal deletion of cells in embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, normal tissue turnover, and a variety of pathologic condition resulting form its dysregulation. A diverse group of signals induce apoptosis including: UV or gamma-irradiation, oxidative damage, chemotherapeutic drugs, growth factor withdrawal, and the cytokines TNF-α and TGF-β. Apoptosis is regulated by two principal pathways.

Cytotoxicity is the cell-killing property of a chemical compound (such as a food, cosmetic, or pharmaceutical) or a mediator cell (cytotoxic T cell). In contrast to

necrosis and apoptosis, the term cytotoxicity does not indicate a specific cellular death mechanism.

For example, cell-mediated cytotoxicity (that is, cell death mediated by either cytotoxic T lymphocytes [CTL] or natural killer [NK] cells) combines some aspects of both necrosis and apoptosis.

1.4.2 Differences between Necrosis and Apoptosis

There are many observable morphological and biochemical differences between necrosis and apoptosis.

Necrosis occurs when cells are exposed to extreme variance from physiological conditions (e.g., hypothermia, hypoxia) which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses.

Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response.

Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue

homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy.

Cells undergoing apoptosis show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed "secondary necrosis".

1.4.3 Apoptotic Pathways

Scientists now recognize that most, if not all, physiological cell death occurs by apoptosis, and that alteration of apoptosis may result in a variety of malignant disorders. Consequently, in the last few years, interest in apoptosis has increased greatly. Great progress has been made in the understanding of the basic mechanisms of apoptosis and the gene products involved.

A Key Elements of the Apoptotic Pathway Include:

Death receptors

Apoptosis has been found to be induced via the stimulation of several different cell surface receptors in association with caspase activation. For example, the CD95

(APO-1, Fas) receptor ligand system is a critical mediator of several physiological and pathophysiological processes, including homeostasis of the peripheral lymphoid compartment and CTL-mediated target cell killing. Upon cross-linking by ligand or agonist antibody, the Fas receptor initiates a signal transduction cascade which leads to caspase-dependent programmed cell death.

Membrane alterations

In the early stages of apoptosis, changes occur at the cell surface and plasma membrane. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell.

Protease cascade

Signals leading to the activation of a family of intracellular cysteine proteases, the caspases, (Cysteinyl-aspartate-specific proteinases) play a pivotal role in the initiation and execution of apoptosis induced by various stimuli. At least 11 different members of caspases in mammalian cells have been identified. Among the best-characterized caspases is caspase-1 or ICE (Interleukin-1C- Converting Enzyme), which was originally identified as a cysteine protease responsible for the processing of interleukin 1β.

Mitochondrial changes

Mitochondrial physiology is disrupted in cells undergoing either apoptosis or necrosis. During apoptosis mitochondrial permeability is altered and apoptosis specific protease activators are released from mitochondria. Specifically, the discontinuity of the outer mitochondrial membrane results in the redistribution of

cytochrome c to the cytosol followed by subsequent depolarization of the inner mitochondrial membrane. Cytochrome C (Apaf-2) release further promotes caspase activation by binding to Apaf-1 and therefore activating Apaf-3 (caspase 9). AIF (apoptosis inducing factor), released in the cytoplasm, has proteolytic activity and is by itself sufficient to induce apoptosis.

DNA fragmentation

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability (prelytic DNA fragmentation). In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+}-dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments.

B The Pathways

The molecular mechanisms of involved in cell death have begun to be elucidated bringing to light a host of proteins involved in the initiation, amplification, and suppression of this process. Apoptosis involves the activation of caspases, key effector enzymes in the death pathway.

Our understanding of the molecular basis of apoptosis is grounded in the seminal work using the nematode *C. elegans*. This work resulted in the identification of three cellular death genes known as *ced-3*, *ced-4*, and *ced-9*. In this nematode, 131 cells are destined to die during development and such death is regulated by the highly conserved *ced-3*, *ced-4*, and *ced-9* genes. *Ced-9* functions as a negative regulator of

apoptosis as a consequence of its interaction with *ced-4* and *ced-3*. The *ced-9* protein is highly homologous to the anti-apoptotic protein Bcl-2. *Ced-3* is a cysteine protease resembling mammalian caspases and serves as an effector molecule to initiate cell death. The *ced-4* protein is highly homologous to mammalian Apaf-1. The proteins *ced-4* and *ced-9* stably interact, by can be displaced by the pro-apoptotic effector Egl-1 (similar to BH3-only domain members of the Bcl-2 family in mammals). In this manner Egl-1 antagonizes the anti-apoptotic function of *ced-9*. The simple, linear, non-redundant model of apoptosis in *C. elegans*, has evolved into a complex, multi-tiered, and redundant model of cell death in mammalian cells.

Extrinsic death pathways

The Fas death pathway serves as a paradigm for mammalian death machinery and is similar to the death signaling induced by TRAIL and TNF receptors. Fas-induced apoptosis plays a critical role in the maintenance of peripheral tolerance and mice with mutations in either the Fas receptor or ligand develop lymphoproliferation and autoimmunity. Engagement of the type II membrane Fas receptor by FasL induces recruitment of the adaptor molecule FADD to the death domain of Fas. This interaction leads to the clustering of pro-caspase 8 at the death-inducing signaling complex (DISC) and caspase-8 activation by proximity. Additional caspases are activated by caspase-8 resulting in the cleavage of essential cellular substrate proteins and the release of caspase activated DNAse (CAD) ultimately resulting in target cell death.

A similar signaling mechanism is observed with the TRAIL receptor, DR4 and DR5, where ligand-induced FADD recruitment to the receptor leads to caspase-8

activation. In contrast to Fas, however, the TRAIL system includes not only the death receptors DR4 and DR5, but also three decoy receptors, DcR1 and DcR2 and OPG (osteoprotegerin). DcR1 lacks a death domain and transmembrane domain, while DcR2 has a nonfunctional death domain. These decoy receptors bind to TRAIL but do not signal, acting to protect cells from TRAIL-induced death. Many tumor cell lines are sensitive to TRAIL-induced apoptosis, suggesting that TRAIL may have therapeutic applications.

Unlike the Fas and TRAIL receptors, signaling through the TNF receptor can result in either cell death or protection against cell death via the activation of NF-κB. NF-κB activation is known to upregulate the expression of the anti-apoptotic proteins cIAP-1, cIAP-2, FLIP, and others. Recent work has provided insight into the underlying biochemical mechanisms resulting in this differential signal. Two separate receptor signaling complexes can be formed when TNFα binds to TNF-R1. The first consists of TRAD, TRAF2, and RIP. This complex activates NF-κB resulting in nuclear translocation and enhanced transcription of pro-survival genes, including $FLIP_L$, and potent caspase-8 inhibitor. In a separate step, RIP and TRADD associate with FADD and caspase 8 in the cytosol, after dissociating from the TNF receptor. Once NF-κB has been activated, this complex also contains $FLIP_L$ with suppresses the induction of cell death. If the first signal for NF-κB activation fails, the apoptosis-inducing complex in the cytosol (lacking $FLIP_L$) signals cell death through caspase activation. A myriad of signals in the microenvironment can also contribute to TNF signaling for death or survival in a particular cell type.

Intrinsic death pathways

Radiation and genotoxic agents activate another death pathway that acts independently of extrinsic death receptors. These agents activate the intrinsic death pathway involving mitochondrial damage to induce cell death. Mitochondrial cell death is regulated by the pro- and anti-apoptotic molecules of the Bcl-2 family. Bcl-2 was first described as a unique oncogene in B cell lymphomas acting to inhibit cell death and induce cellular proliferation. Since the identification of Bcl-2, numerous family members have been identified, including the pro-survival proteins Bcl-X_L and Mcl-1, and the pro-apoptotic proteins Bid, Bax, and Bak (and Egl-1 in *C. elegans*). Studies have shown that Bax and Bak are critically required for mitochondrial regulated cell death in mammals. Cells deficient in both Bax and Bak are resistant to many apoptotic stimuli that induce death through mitochondrial disruption. In the mitochondria, inactive Bak resides in a complex VDAC2. Upon translocation of pro-death molecules to the mitochondria, VDAC2 is displaced and Bak is activated. Translocation of mammalian pro-apoptotic proteins to the mitochondria also allows antagonistic interaction with Bcl-2 to suppress its pro-survival function.

The p53 tumor suppressor induces apoptosis through the induction of pro-apoptotic proteins. p53 is often mutated or deleted in cancer cells and has been shown to function both in cell cycle arrest and induction of apoptosis, depending on the cellular context. p53 transcriptionally regulates proteins involved in mitochondrial cell death (Bax, Noxa, and PUMA). In addition, p53 transcriptionally activates extrinsic death receptor promoters including Fas and TRAIL and acts to increase protein export to the surface.

The extrinsic and intrinsic pathways intersect in some cell types through the cleavage of Bid, and pro-apoptotic Bcl-2 family member, by activated caspase-8. Bid cleavage results in translocation to the mitochondria and interaction with Bax and Bak to induce mitochondrial damage. Bid cleavage by caspase-8 links the intrinsic pathway to the death receptor-induced signal.

The pro-apoptotic molecules Bax, Bak, Noxa, Bid, and others can be regulated, through localization and modification, to induce apoptosis by forming pores in the mitochondria. Once inserted, the pro-apoptotic proteins destabilize mitochondrial membrane integrity (Bcl-2 family pro-survival proteins act to stabilize mitochondrial membrane integrity) and allow cytochrome c release from the mitochondria. Cytosolic cytochrome c binds the adapter molecule Apaf-1, leading to recruitment of inactive pro-caspase 9, the initiator caspase of the intrinsic pathway. When caspase 8 or 9 procaspases are brought into proximity with one another through their respective adapter proteins, self-activation occurs. Recent work has shown that it is the dimerization of inactive pro-caspases that leads to activation - internal proteolysis as a secondary event of dimerization-induced activation of the monomeric zymogens. Ultimately, downstream caspases (such as caspase 3) re activated, leading to many of the hallmark features of apoptosis, including DNA fragmentation and PARP cleavage.

Caspase activity can be modulated by dominant negative caspases, such as FLIP, (a caspase-8 dominant negative molecule), and by caspase-binding proteins of the IAP family. Overexpression of these pro-survival molecules, including XIAP, survivin, and FLIP is observed in many human tumors. Additionally, these caspase inhibitors can be antagonized by mitochondrial release of Smac/DIABLO.

Smac/DIABLO can displace XIAP from its interaction with activated caspase 3, allowing for cell death. Thus, both the activation and function of caspases can be regulated through multiple binding proteins.

Another mitochondrial component with a regulatory role in apoptosis is AIF, or apoptosis-inducing factor. AIF has homology to bacterial oxidoreductases and is normally localized in the mitochondria, but translocates to the cytoplasm during apoptosis. Apoptosis induced by AIF cannot be inhibited by the caspase inhibitors, and can result in nuclear condensation and DNA fragmentation independent of other pro-apoptotic factors. AIF also affects the mitochondria allowing a positive feedback loop and enhanced apoptosis. Interestingly, the redox-active enzymatic region of AIF is anti-apoptotic, giving the molecule a dual role in cell survival and cell death.

The end result of apoptosis is to render cell death in the absence of inflammation. Apoptotic bodies are recognized and consumed by phagocytic cells and through the interaction of scavenger receptors such as CD14 and CD36 with exposed phosphatidylserine on the outer leaflet of the apoptotic cell membrane.

1.4.4 Apoptosis Assay Methods

Originally, to study both forms of cell death, necrosis and apoptosis, cytotoxicity assays were used. These assays were principally of two types:

• Radioactive and non-radioactive assays that measure increases in plasma membrane permeability, since dying cells become leaky.

• Colorimetric assays that measure reduction in the metabolic activity of mitochondria; mitochondria in dead cells cannot metabolize dyes, while mitochondria in live cells can.

However, as more information on apoptosis became available, researchers realized that both types of cytotoxicity assays vastly underestimated the extent and timing of apoptosis. Although the cytotoxicity assays might be suitable for detecting the later stages of apoptosis, other assays were needed to detect the early events of apoptosis.

With increased understanding of the physiological events that occur during apoptosis, a number of assay methods have been developed for its detection. For instance, these assays can measure one of the following apoptotic parameters:

• Fragmentation of DNA in populations of cells or in individual cells, in which apoptotic DNA breaks into different length pieces.

• Alterations in membrane asymmetry. Phosphatidylserine translocates from the cytoplasmic to the extracellular side of the cell membrane.

• Activation of apoptotic caspases. This family of proteases sets off a cascade of events that disable a multitude of cell functions.

• Release of cytochrome C and AIF into cytoplasm by mitochondria.

A Methods for Studying Apoptosis in Cell Populations

A number of methods have now been developed to study apoptosis in cell populations. We focus on two key apoptotic events in the cell:

(1) Apoptosis and cell mediated cytotoxicity are characterized by cleavage of the genomic DNA into discrete fragments prior to membrane disintegration. Because

DNA cleavage is a hallmark for apoptosis, assays which measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death. The DNA fragments may be assayed in either of two ways:

• As “ladders” (with the 180 bp multiples as “rungs” of the ladder) derived from populations of cells.

• By quantification of histone complexed DNA fragments with an ELISA.

(2) Researchers discovered that proteases were involved in the early stages of apoptosis. The appearance of these caspases sets off a cascade of events that disable a multitude of cell functions. Caspase activation can be analyzed in different ways:

• By an *in vitro* enzyme assay. Activity of a specific caspase, for instance caspase 3, can be determined in cellular lysates by capturing of the caspase and measuring proteolytic cleavage of a suitable substrate.

• By detection of cleavage of an *in vivo* caspase substrate. For instance caspase 3 is activated during early stages. Its substrate PARP (Poly-ADP-Ribose-Polymerase) and the cleaved fragments can be detected with the anti PARP antibody.

Assays that measure DNA fragmentation

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die. In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+}- dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments. These DNA fragments reveal, upon agarose gel

electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit.

Radioactive as well as non-radioactive methods to detect and quantify DNA fragmentation in cell populations have been developed. In general, these methods are based on the detection and/or quantification of either low molecular weight (LMW) DNA which is increased in apoptotic cells or high molecular weight (HMW) DNA which is reduced in apoptotic cells. The underlying principle of these methods is that DNA, which has undergone extensive double-stranded fragmentation (LMW DNA) may easily be separated from very large, chromosomal length DNA (HMW DNA), e.g., by centrifugation and filtration.

For the quantification of DNA fragmentation, most methods involve a step in which the DNA of the cells has to be labeled: Prior to the addition of the cell death-inducing agent or of the effector cells, the (target) cells are incubated either with the [^{3}H]- thymidine ([^{3}H]-dT) isotope or the nucleotide analog 5-bromo-2'-deoxyuridine (BrdU). During DNA synthesis (DNA replication) these modified nucleotides are incorporated into the genomic DNA. Subsequently, those labeled cells are incubated with cell death-inducing agents or effector cells and the labeled DNA is either fragmented or retained in the cell nucleus. Finally each type of DNA (HMW and LMW) is quantitated. Because the labeling of the cellular DNA has to be done prior to the induction of cell death, this labeling is also called "prelabeling".

The prelabeling of one cell population (e.g., the target cells) allows the behavior of the labeled cells to be traced specifically when different cell populations are mixed.

In a study of cell-mediated cytotoxicity the target cell population is labeled before the effector cells (e.g., CTL) are added. Subsequently, due to pore formation in the target cell plasma membrane, the fragmented LMW DNA is released from the cytoplasm of the target cell into the culture supernatant. The cytotoxic potential of the effector cells is measured by quantification of the label released from the damaged target cells.

Because this metabolic prelabeling of the genomic DNA requires DNA synthesis, only cells proliferating *in vitro* (e.g., cell lines) may be labeled in this way; cells which do not proliferate *in vitro* (e.g., primary cell cultures, tumor cells *ex vivo*) do not replicate their DNA and therefore, do not incorporate labeled nucleotides.

To detect fragmented DNA in cells which do not replicate *in vitro*, the DNA has to be isolated and analyzed by agarose gel electrophoresis.

An alternative method which circumvents the isolation and electrophoretic analysis of DNA is the immunological detection of LMW DNA (histone-complexed DNA fragments) by an immunoassay.

Each of the methods to detect and measure apoptosis has its advantages and limitations. Because the cellular mechanisms that result in apoptosis are complex, most published methods cannot by themselves detect apoptosis unambiguously.

To ensure that the mode of cell death in the individual cell system or experiment is apoptotic, one also has to consider other criteria like the cellular morphology. Morphologic criteria for apoptotic cell death include, for example, chromatin condensation with aggregation along the nuclear envelope and plasma membrane blebbing followed by separation into small, apoptotic bodies. When

internucleosomal DNA fragmentation is accompanied by these morphological features it provides an additional useful criterion to define cell death as apoptotic.

Assays that measure apoptosis induced proteases (caspases)

Several caspases are thought to mediate very early stages of apoptosis. For instance, one of these, caspase 3 (CPP32) is required for the induction of apoptosis by certain effectors [especially tumor necrosis factor and the cytotoxic T cell ligand effector, CD95 (also called Fas)]

These proteases cleave numerous substrates at the carboxyl site of an aspartate residue. All are synthesized as pro-enzymes; activation involves cleavage at aspartate residues that could themselves be sites for the caspase family. As caspases are probably the most important effector molecules for triggering the biochemical events which lead to apoptotic cell death, assays for determination of caspase activation can detect apoptosis earlier than many other commonly used methods.

The most elucidatory assay for these caspases involves western blot detection of proteolytic cleavage products found in apoptotic cells. An antibody, Anti-PARP, can be used in such an assay. The antibody can detect intact and cleaved forms of Poly-ADP Ribose Polymerase, a target for some caspases.

For specific and quantitative measurement of caspase activity Western blotting is not suitable. To quantify caspase activation enzyme activity assays based on detection of cleaved caspase substrates have been developed recently. However most of the caspase substrates are not exclusively cleaved by a specific caspase but only preferentially, while other members of the caspases family act on these substrates to a lower extent.

B Methods for Studying Apoptosis in Individual Cells

We focus on two key apoptotic events in the cell:

(1) DNA fragmentation used to study death in cell populations may also be used to study death in individual cells. DNA cleavage is a hallmark for apoptosis, and assays which measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death.

The methods used to assess DNA strand breaks are based on labeling/ staining the cellular DNA. The labeled/ stained DNA is subsequently analyzed by flow cytometry, fluorescence microscopy or light microscopy. In general, two different labeling methods may be used to identify DNA in apoptotic cells:

• Enzymatic labeling: Cellular DNA is labeled with modified nucleotides (e.g., biotin-dUTP, DIG-dUTP, fluorescein-dUTP) using exogenous enzymes (e.g., terminal transferase, DNA polymerase). This labeling detects extensive DNA strand breaks.

• Staining with fluorochromes: Cellular DNA is stained with fluorescent DNA-binding dyes (DNA fluorochromes) capable of intercalating into DNA. Upon binding to DNA these dyes become highly fluorescent. Apoptotic cells are binding less dye molecules, since they characteristically lose DNA during the staining process.

(2) In addition, individual cell death may be studied by assays that measure alterations in plasma membranes (alterations in the asymmetry or permeability of individual cell membranes, which occur as the membrane shrinks and becomes increasingly convoluted.) For instance, during apoptosis, phosphatidylserine translocates from the cytoplasmic side of the membrane to the extracellular side and can be detected with Annexin V.

The TUNEL enzymatic labeling assay

Extensive DNA degradation is a characteristic event which often occurs in the early stages of apoptosis. Cleavage of the DNA may yield double-stranded, LMW DNA fragments (mono- and oligonucleosomes) as well as single strand breaks ("nicks") in HMW-DNA. Those DNA strand breaks can be detected by enzymatic labeling of the free 3'-OH termini with modified nucleotides (X-dUTP, X = biotin, DIG or fluorescein). Suitable labeling enzymes include DNA polymerase (nick translation) and terminal deoxynucleotidyl transferase (end labeling). DNA polymerase I catalyzes the template dependent addition of nucleotides when one strand of a double-stranded DNA molecule is nicked. Theoretically, this reaction (*In Situ* Nick Translation, ISNT) should detect not only apoptotic DNA, but also the random fragmentation of DNA by multiple endonucleases occurring in cellular necrosis.

Terminal deoxynucleotidyl transferase (TdT) is able to label blunt ends of doublestranded DNA breaks independent of a template. The end-labeling method has also been termed TUNEL (TdT-mediated XdUTP nick end labeling).

The TUNEL method is more sensitive and faster than the ISNT method. In addition, in early stages cells undergoing apoptosis were preferentially labeled by the TUNEL reaction, whereas necrotic cells were identified by ISNT. Thus, experiments suggest the TUNEL reaction is more specific for apoptosis and the combined use of the TUNEL and nick translation techniques may be helpful to differentiate cellular apoptosis and necrosis.

To allow exogenous enzymes to enter the cell, the plasma membrane has to be permeabilized prior to the enzymatic reaction. To avoid loss of LMW DNA from the permeabilized cells, the cells have to be fixed with formaldehyde or glutaraldehyde before permeabilization. This fixation crosslinks LMW DNA to other cellular constituents and precludes its extraction during the permeabilization step.

If free 3' ends in DNA are labeled with biotin- dUTP or DIG-dUTP, the incorporated nucleotides may be detected in a second incubation step with (strept)avidin or an anti- DIG antibody. The immunocomplex is easily visible if the (strept)avidin or an anti- DIG antibody is conjugated with a reporter molecule (e.g., fluorescein, AP, POD).

In contrast, the use of fluorescein-dUTP to label the DNA strand breaks allows the detection of the incorporated nucleotides directly with a fluorescence microscope or a flow cytometer. Direct labeling with fluorescein-dUTP offers several other advantages. Direct labeling produces less nonspecific background with sensitivity equal to indirect labeling and, thus, is as powerful as the indirect method in detecting apoptosis. Furthermore, the fluorescence may be converted into a colorimetric signal if an anti-fluorescein antibody conjugated with a reporter enzyme is added to the sample.

Assays that measure membrane alterations

In contrast to necrosis, apoptosis occurs without inflammation. In the end stages of apoptosis, apoptotic bodies are engulfed by macrophages and other phagocytic cells *in vivo*. Thus, apoptotic cells are removed from the population without spilling their contents and eliciting an inflammatory response.

The exact mechanism by which the apoptotic cell becomes a target for phagocytes is unclear. However, it has been shown that a number of changes in cell surface (membrane) markers occur during apoptosis, any one of which may signal "remove now" to the phagocytes. These membrane changes include:

• Loss of terminal sialic acid residues from the side chains of cell surface glycoproteins, exposing new sugar residues.

• Emergence of surface glycoproteins that may serve as receptors for macrophage secreted adhesive molecules such as thrombospondin.

• Loss of asymmetry in cell membrane phospholipids, altering both the hydrophobicity and charge of the membrane surface.

In theory, any of these membrane changes could provide an assay for apoptotic cells. In fact, one of them has – the alteration in phospholipid distribution.

In normal cells, the distribution of phospholipids is asymmetric, with the inner membrane containing anionic phospholipids (such as phosphatidylserine) and the outer membrane having mostly neutral phospholipids. In apoptotic cells however, the amount of phosphatidylserine (PS) on the outer surface of the membrane increases, exposing PS to the surrounding liquid.

Annexin V, a calcium-dependent phospholipid- binding protein, has a high affinity for PS. Although it will not bind to normal living cells, Annexin V will bind to the PS exposed on the surface of apoptotic cells. Thus, Annexin V has proved suitable for detecting apoptotic cells.

Assays that use DNA stains

One can differentiate between three methods for studying cell death that use DNA stains: dye exclusion method, profile of DNA content, morphological changes.

Dye exclusion method

Viable (intact plasma membrane) and dead (damaged plasma membrane) cells can be discriminated by differential staining. Cells with disturbed plasma membrane permeability are stained, whereas undamaged (viable) cells are not stained with dyes that do not penetrate the plasma membrane ("exclusion dyes"). The most frequently used dye for exclusion tests is trypan blue. In addition, the fluorescent dye, propidium iodide (PI) which becomes highly fluorescent after binding to DNA, can be used in the same manner. The stained and unstained cells are counted with a standard light microscope (trypan blue), or flow cytometer (PI).

• **Annexin V-propidium iodide (PI) double stain**

The Annexin-V binding assay is based on the relocation of phosphatidylserine to the outer cell membrane. Viable cells maintain an asymmetric distribution of different phospholipids between the inner and outer leaflets of the plasma membrane. Choline-containing phospholipids such as phosphatidylcholine and sphingomyelin are primarily located on the outer leaflet of viable cells and aminophospholipids such as phosphatidylethanolamine and phosphatidylserine (PS) are found at the cytoplasmic (inner) face of viable cells. The distribution of phospholipids in the plasma membrane changes during apoptosis. In particular, PS relocates from the cytoplasmic face to the outer leaflet so called PS exposure. The extent of PS exposure can distinguish apoptotic cells from the non-apoptotic cells. Annexin-V is a 35-36 kDa calcium-

dependent phospholipid binding protein with high affinity for PS (kDa ~ 5x10-10M). When labeled with a fluorescent dye, Annexin-V can be used as a sensitive probe for PS exposure on the outer leaflet of the cell membrane.

The binding of Annexin-V conjugates such as Annexin-V FITC to cells permits differentiation of apoptotic cells (Annexin-V positive) from non-apoptotic cells (Annexin-V negative). Annexin-V binding is observed under two conditions. The first condition is observed in cells midway through the apoptosis pathway. Phosphatidylserine translocates to the outer leaflet of the cell membrane. The second condition is observed in very late apoptosis or when the cells become necrotic and membrane permeabilization occurs. This membrane permeabilization allows Annexin-V to enter cells and bind to phosphatidylserine on the cytoplasmic face of the membrane. Since other causes besides apoptosis can result in necrosis, it is important to distinguish between necrotic and apoptotic cells. Membrane permeabilization also permits entry of other materials to the interior of the cell, including the fluorescent DNA-binding dye propidium iodide. Utilizing dual staining methodology, apoptotic populations can be distinguished from necrotic populations. For example, using the Annexin V-propidium iodide (PI) double staining regime (Figure 4.2.1), three populations of cells are distinguishable in two-color flow cytometry.

Profile of DNA content

If cells are permeabilized, the LMW DNA inside the cytoplasm of apoptotic cells leaks out during the subsequent rinse and staining procedure. The lower DNA content of these cells means they contain less DNA stained by the fluorochrome. Thus, cells with lower DNA staining than that of G1 cells (the so-called “sub-G1

peaks", "A0" cells) have been considered apoptotic. The reduction in staining/DNA content of these cells is measured by flow cytometry. The major disadvantage of this technique is that apoptotic G2-Phase cells exhibit a reduced DNA content, which could represent the DNA content of a G1- cell. Therefore it may not be detected as apoptotic. This would result in an underestimation of the apoptotic population.

Morphological changes

On the other hand, the bisbenzimidazole dye, Hoechst 33342 (and also acridine orange), penetrates the plasma membrane and stains DNA in cells; without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy. They are also visible in permeabilized apoptotic cells stained with other DNA binding dyes like DAPI.

One drawback of using any vital staining method for measuring apoptosis is the variability of active dye uptake in different cells and its possible change during certain treatments. Therefore, the ability of Hoechst 33342 to discriminate apoptotic cells from normal cells by increased uptake of dye has to be tested for each new cell system..

C Detection of Apoptosis-Related Proteins

There are a number of genes that regulate apoptosis. That is, the products of these genes interfere with apoptotic pathways. Assays to detect the proteins encoded by these genes can complement the assays described in the previous sections.

The study of apoptosis-regulating genes and gene products is still evolving. The picture so far is complex. For instance, in some cases, the same gene has an effect on both the survival of normal cells and the development of cancers by preventing apoptosis. A detailed discussion of the field is beyond the scope of this guide, but is covered in a number of reviews.

In mammalian systems, the Bcl-*2* proto-oncogene serves much the same function as ced-9, blocking the induction of cell death41. The Bcl-2 oncoprotein also protects against the cytotoxic effects of certain drugs.

The Bcl-2 protein can dimerize with itself or with the product of the bax gene. The presence of the bax protein seems to counteract the anti-apoptotic activity of Bcl-2. In summary, apoptosis is more likely when bax protein levels are high or when Bcl-2 protein levels are low.

Another mammalian gene product, p53, is a tumor suppressor because it induces apoptosis in potentially malignant cells. Absence or mutation of the p53 gene product led to malignant transformation and immortalization of the cell.

Increases in expression of a growth stimulating gene, the c-myc proto-oncogene, actually induces apoptosis in the absence of other growth factors. High levels of the Bcl-2 protein can counteract the effect of the c-myc protein.

Cell surface receptor genes (APO-1/Fas/ CD95), other growth-stimulating genes (e.g., Ras), and other tumor-suppressing genes (e.g., Rb) have also been implicated in the regulation of apoptosis. The Fas (CD95/APO-1) molecule has originally been identified as a cell surface receptor that could mediate apoptotic cell death of transformed cells and cause regression of experimental tumors growing in nude mice. The function of Fas was assessed by establishment of mouse cell transformants that constitutively expressed human Fas. When the transformed cells were treated with the antibody to human Fas, the cells died by apoptosis within 5 hours, which indicated that Fas can transduce an apoptotic signal and that anti-Fas works as an agonist. The subsequent purification of human APO-1 antigen and molecular cloning of its cDNA established the identity of APO-1 and Fas. Meanwhile, numerous reports have shown a pivotal role of Fas in various physiological and pathological processes.

1.4.5 Apoptosis in Hypertension

Recently, a number of studies suggest an elevated level of cell apoptosis in the thymus, brain, kidney, smooth muscle, and cardiac muscle of the SHR (11, 13, 14, 17, 30, 34, 39, 45) as well as capillary endothelial apoptosis in a one kidney/one clip (1K1C) Goldblatt model of hypertension (15). Also, in the 1K1C Goldblatt model, Gobé et al. showed apoptotic bodies (with cytoplasm and nuclei) in shrunken capillaries, which were often seen, were often surrounded by collagen fragments, indicating basement membrane breakdown (15).

Using TUNEL labeling technique, the result of my pilot study indicated that SHR thymus has elevated apoptosis level, and lower cell number density (Figure 1.4.5).

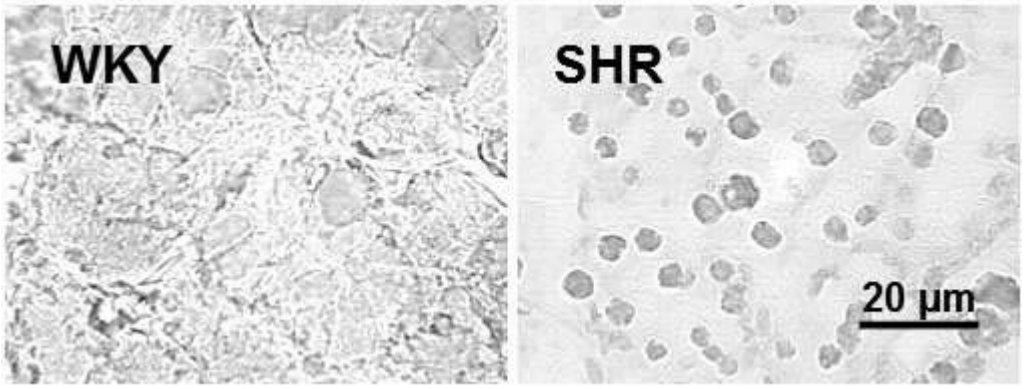

Figure 1.4.5 Micrographs of TUNEL labeling in thymus of WKY and SHR.

1.5 Leukocyte Capillary Plugging

1.5.1 Capillary

Capillaries are the thinnest vessels, functioning to allow transport through their walls to and from the blood and tissues. The wall of a capillary consists of a single layer of endothelial cells surrounded by a basement membrane, which encloses pericytes. The endothelial cells of the capillary wall have their edges closely contacted to one another. Junctional complexes seal the intercellular clefts at certain points. Capillaries come in several types:

• Continuous capillaries are formed of a single layer of simple squamous epithelium, a continuation of the endothelial lining, held together by tight junctions allowing only small molecules to pass. They are found in the brain, muscles, mesentery, the skin, the lungs and many other organs.

• Fenestrated capillaries have pores to increase transport. They are found in the absorptive capillaries in the GI tract, in glands, in the glomerulus of kidneys, etc.

• Sinusoids are chamber-like vessels which allow blood from several sources to mix. They are found in the liver, spleen, and lymph nodes.

1.5.2 Leukocyte

A Properties

Leukocytes, also called white blood cells, are an important part of the body's immune system. They protect the body against invading bacteria, parasites, and

viruses by killing these microorganisms through phagocytosis (ingestion) and other antigen-specific cytotoxic mechanisms. There exist five different types of leukocytes (neutrophils, eosinophils, basophils, monocytes, and lymphocytes), each of which is designed to fight a specific type of infection. These cells are recruited from the circulation to the sites of infection by the process called leukocyte adhesion cascade. There are five stages in this process.

(1) A leukocyte contacts the activated endothelium of blood vessels near the infected site. Adhesion results if selectins on endothelial cells bind to the corresponding receptors on the leukocyte.

(2) This interaction mediates rolling of the leukocyte along the endothelium. Otherwise the cell continues to move at the hydrodynamic velocity.

(3) Chemoattractant molecules (chemokines) expressed on the activated endothelium trigger the activation of integrins on the leukocyte membrane, which become high affinity receptors. Selectin binding to its ligand also activates integrin recruitment and affinity changes. Integrins interact with corresponding ligands on the endothelial cells. As a result, rolling of the leukocyte slows down.

(4) The leukocyte comes to a stop and attaches firmly to the endothelium by integrin bonds and through the cell activation: the cell is significantly deformed and forms projections or pseudopods

(5) In the active state, the leukocyte transmigrates across the endothelium and moves toward infection. In some cases leukocytes can adhere via attachment to another leukocyte attached to the endothelium, a process known as secondary adhesion.

Receptor-mediated leukocyte adhesion has been studied theoretically either by analytical or semianalytical models in which the leukocyte is a three-dimensional (3D) rigid cell or by numerical 2D models wherein the leukocyte deformability is taken into account. Only recently, the adhesive dynamics of nondeformable spherical particles has been examined numerically on the basis of the 3D boundary integral method.

B Viscoelasticity

Leukocytes have a more complex structural organization than other blood cells. In comparison with erythrocytes (red blood cells), white blood cells contain the nucleus, which is characterized by a low deformability, a complex cytoplasm with many organelles, and the cytoskeleton, which ties together the cell membrane, cytoplasm, and nucleus. Leukocytes are less deformable than erythrocytes and conserve their spherical shape when they flow freely in large blood vessels. However, these cells (with diameter of 8 mm and more) undergo significant deformation when entering the smallest blood capillaries. *In vivo* experiments show that the adherent leukocytes deform to a teardrop shape during rolling along the endothelium. *In vitro*, leukocytes behave as viscoelastic liquid drops when aspirated into a micropipette. From the top view, it is very difficult to observe shape changes in the rolling cell, especially near the contact area between the cell and the adhesive surface. Further, the velocities at which leukocytes roll *in vitro* and *in vivo* tend to plateau with increasing the wall shear stress. The increased adhesion at higher values of the wall shear stress is due to greater leukocyte deformation.

The deformation of leukocytes in a narrow vessel is showed in figure 1.5.2. The effect of cytoplasmic viscoelasticity on the shapes of inactivated leukocytes makes the cells able to pass through the narrow channel.

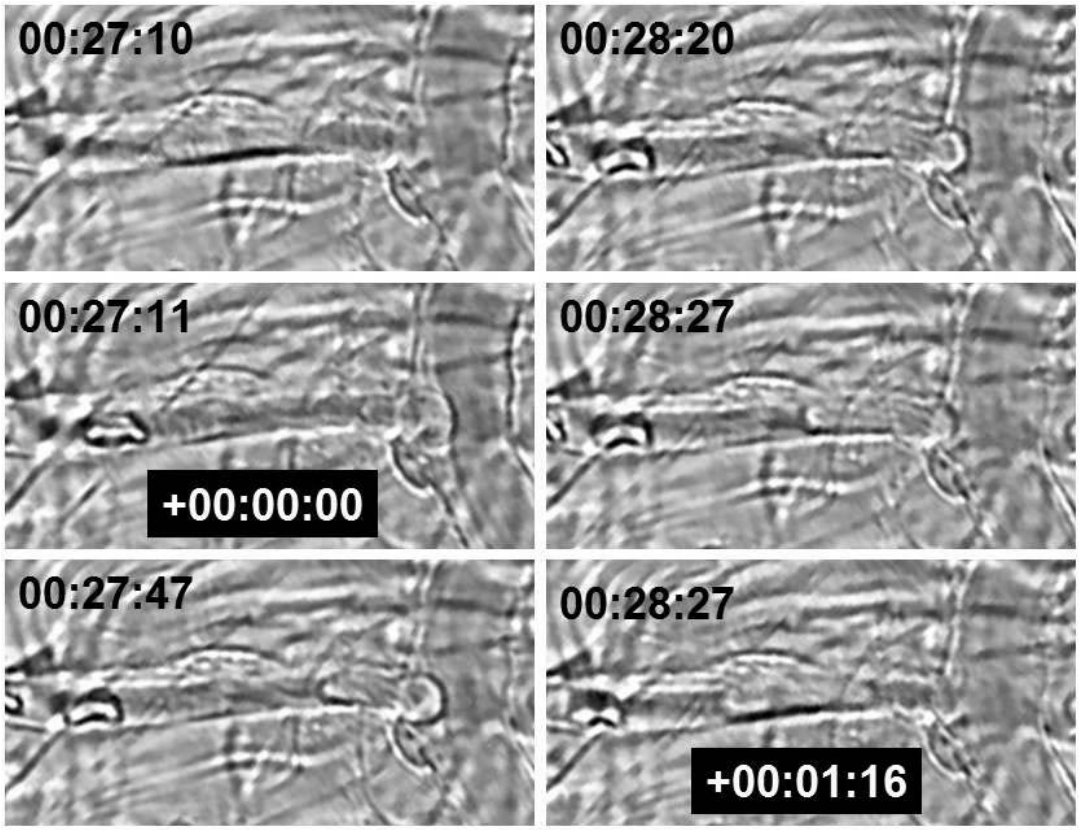

Figure 1.5.2 The deformation of leukocytes in a narrow vessel. A leukocyte (~ 8 μm) slowly deform and pass through a narrow capillary (~ 4μm).

The capillary permanent stasis can happen if the vessel is plugged by either an activated leukocyte or firmly attached leukocyte on the endothelium. If activated, the cytoplasmic of leukocyte becomes plastic, and the cell will no longer deform infinitively to pass through the narrow channel, and the vessel will develop permanent stasis. If strong receptor-ligand bonds can be formed between a leukocyte and the endothelium, the leukocyte will obstruct the vessel.

1.5.3 Leukocyte Plugging

A Intravital Observation of Normal Flow

Typical capillaries in this study were about 8 to 10 μm in diameter with a single file of erythrocytes and regular passage of leukocytes and platelets. Individually blood cells became clearly visible at reduced blood velocity. In the mesentery parenchyma mast cells, fibroblasts, mesenchymal cells, and networks of collagen bundles were visible at the optical resolution employed in the current experiments. At the time the mesentery was exposed, there was normal blood flow in all capillaries (Figure 3.3.2 and Figure 4.4.1A and B at 10 min). Blood flow in individual capillaries may accelerate, decelerate, stop, or even temporarily reverse.

B Cell Plug

In normotensive and in acute ischemia it has been shown that leukocytes may obstruct capillaries (stasis) due to their relative low cell deformability and ability to adhere to endothelium (40).

Minimal endothelial damage and only scarce platelet adhesion evoke significant colocalization of leukocytes (55).

My pilot study indicated that in Wistar the fraction of leukocytes plugging capillaries was less than 1% even under low flow velocity, whereas capillary plugging in DEX-Wistar was significantly more frequent at reduced flow rates. Some capillaries were obstructed by leukocytes from DEX-Wistar. In SHR, most capillaries were easily occluded during reduced flow rates so that the fraction of leukocytes plugging capillaries could not be counted.

After all, I have proposed links between apoptosis, rarefaction, and stasis.

1.6 Hypothesis

I proposed that there are three consequent steps lead to capillary rarefaction in hypertension:

1. Elevated level of apoptosis of capillary endothelial cells in SHR leads to fewer endothelial cells along the capillary network.

2. Adhesion of leukocytes to the endothelium leads to capillary stasis.

3. The capillary stasis leads to quick progression of endothelial apoptosis, the first step of capillary rarefaction.

To make it short: Elevated Level of Apoptosis – Stasis – More Apoptosis
While apoptosis may be a plausible hypothesis for rarefaction, to date the majority of studies on apoptosis in the microcirculation have relied on histological or morphological evidence derived from still images. No approach has been proposed that serves to document the in-vivo events in a living microcirculation during

apoptosis so that the microvascular events that lead to rarefaction remain speculative. To study microvascular cell death and capillary rarefaction in-vivo, we developed a method for detection of endothelial cell and parenchymal cell apoptosis in a living tissue, while recording at the same time the events in the microcirculation. Besides central pressure, cell death and capillary stasis are surely dependent on humoral factors, extracellular matrix and cellular interactions between endothelial cells, platelets, and leukocytes.

2 Objectives

2.1 Objective

This dissertation is concerned with an analysis of microvascular mechanisms, which result in the progressive organ injury in hypertension. Specifically, the objective is to define cellular mechanisms responsible for microvascular rarefaction in the SHR and WKY rat after acute dexamethasone application.

2.2 Specific Aims

In microcirculation of the animal models of hypertension,

Aim 1. To analyze the correlation between capillary stasis and endothelial apoptosis

Aim 2. To determine the cellular events leading to capillary stasis.

2.3 Significance

1. The work will lead to defined cellular mechanisms responsible for enhanced microvascular complications in hypertension.

2. The knowledge from hypertension study could apply for other cardiovascular diseases.

3. This may be an important target for new pharmaceutical therapies for hypertensives.

3 Specific Aim 1. The Correlation between Capillary Stasis and Endothelial Apoptosis

Hypothesis: capillary stasis plays an important role in endothelial apoptosis

The first specific aim is to analyze the correlation between capillary stasis and endothelial apoptosis, with the following sub-aims:

Sub-Aim 1a. To analyze the correlation between incidence of stasis and capillary endothelial cell density

Sub-Aim 1b. To analyze the correlation between stasis and endothelial cell death

3.1 Introduction: Effect of Glucocorticoid on Endothelial Cells

The five-day DEX-treated Wistar rats exhibit a 10% increase in cell death along the mesenteric arteriolar and venular endothelium compared with controls (53).

After adrenalectomy, no significant differences in cell death remained between WKY rats and SHRs.

With five-day DEX treatment, the adrenalectomized SHRs had a higher number of apoptotic endothelial cells than the WKY rats (29).

3.2 Materials and Methods

3.2.1 Animals

The experimental protocol was reviewed and approved by the University of California San Diego Animal Subjects Committee. Male SHRs, and normotensive

WKY rats (Charles River Laboratories, Wilmington, MA, USA) at 12-18 weeks of age (280-350 g) were anesthetized with sodium nembutal (50 mg/kg body weight, Abbott Laboratories, North Chicago, IL, USA, i.m.). Polyethylene (PE) catheters (PE50, I.D. 0.5 mm/ O.D. 0.956 mm, Becton Dickinson Primary Care Diagnostics, Sparks, MD) were placed into the femoral artery and femoral vein prior to start of surgery. The mean arterial pressure and heart rate were recorded by a laboratory computer (Power Macintosh G3 with MacLab, Apple Computer Company, Cupertino, CA). Supplemental doses of anesthesia were administered intravenously at a dose of 5 mg/kg as needed after reflex testing. Body temperature was maintained at 37°C by a water-heated animal stage. At the end of the study, the animals were euthanized (pentobarbital 120 mg/kg body weight, i.v.).

3.2.2 Mesentery Preparation

The abdominal cavity was exposed via a mid-line incision. The intestinal mesentery was withdrawn and loosely draped over an observation window on the heated animal stage by carefully manipulating the intestine with cotton-tipped applicators. To avoid drying and hyperosmotic injury, the exposed intestine and adjacent mesenteric sections were covered with sponge gauze and kept continuously superfused with Krebs-Henseleit solution (pH 7.4, 305 mOsm, 37°C).

3.2.3 Reagents

Fluorescein-Labeled Annexin V (FITC-annexin V, Invitrogen, Carlsbad, CA; excitation at 490 nm and emission at 520 nm), a phospholipid-binding protein with high affinity for phosphatidylserine at the cell surface, and was used for identifying early-stage apoptotic cells with exposed phosphatidylserine.

Propidium Iodide (PI, 1 μM, Sigma Chemical Company, St. Louis, MO; excitation at 536 nm and emission at 617 nm), a fluorescent probe that can only enter cells with membrane damage and that binds nucleic acids (26, 35), was used to label and quantify the level of microvascular endothelial cell death and parenchymal cell death (at late-stage apoptosis or necrosis) (42).

Dexamethasone (DEX, 1 μg/ml or 2.55 μM, Sigma), as a synthetic hormone with 25 times the glucocorticoid activity of cortisol and little mineralocorticoid activity, was used to examine the effects of glucocorticoids (54). DEX was injected i.p. and superfused onto the exposed mesentery, a procedure that serves to enhance apoptosis.

Monastral Blue (3% suspension of pigment in 0.85% NaCl, Sigma) was injected intravenously (30 mg/kg body weight) to identify sites of increased vascular permeability.

Eptifibatide (Integrilin, Millennium Pharmaceuticals, Inc.) binds to the platelet receptor glycoprotein (GP) IIb/IIIa and inhibits platelet aggregation. Eptifibatide was intravenously administered as two 180 μg/kg body weight boluses 10 minutes apart and a continuous infusion of 2.0 μg/kg body weight /min.

Fucoidin (7 mg/kg body weight, 166 kDa, Sigma), a polysaccharide composed predominantly of sulfated fucose, binds to L-selectin and inhibit L-selectin-mediated lymphocyte adhesion and also inhibit leukocyte rolling (28).

Anti L-selectin antibody (Lam1-116, 45 μg/kg body weight, Santa Cruz Biotechnology, Santa Cruz, CA) is a mouse monoclonal IgG2a antibody raised against the lectin domain of L-selectin of mouse origin.

3.2.4 Intravital Microscopic Observation of Microcirculation

The mesentery microcirculation was viewed with a 40X water-immersion lens (numerical aperture 0.75, Zeiss, Oberkochen, Germany) on an intravital microscope (Leitz, Wetzlar, Germany). This resolution is a compromise between a high resolution required to see individual platelets and lower magnification to observe longer segments of capillaries and the location where capillary closures occur. Bright-field and fluorescent images were recorded with a color charge-coupled device camera (Model VI-470, Optronics, Goleta, CA) and stored on a video recorder (Model VC-S101, Sharp, Malaysia). Selected tissue areas were videotaped for the full duration of the experiment. The intravital microscopic observation readily permits detection of blood flow stasis, a change in cell morphology, cellular interaction, and other events in blood vessels and tissue parenchyma. Fluorescent field images show nuclei of PI-positive cells and their morphology (Figure 1).

3.2.5 Experimental Protocol

WKYs and SHRs were subdivided into control and DEX-treated groups. The DEX-treated groups were administered 5 ml of DEX solution (1 μg/ml in saline, pH 7.4, 37°C, i.p.), and the control groups with 5 ml saline (pH 7.4, 37°C, i.p.). In order to maintain an enhanced DEX concentration in the abdominal cavity, another 5 ml solution was injected (i.p.) after one hour to supplement the initial dose of DEX. After a two-hour exposure to DEX or saline in the controls, the intestinal mesentery was exteriorized. The mesentery and the small intestine were kept continuously superfused with Krebs-Henseleit solution with PI (1 μM), and DEX (1 μg/ml) in the case of the DEX-treated groups. Bright-field and simultaneous fluorescent images of selected mesenteric capillaries and their surrounding tissue parenchyma were recorded. The exposure time to fluorescent light was kept below 10 seconds per image with one image per 15-minute interval for a total observation period of three hours. Pilot experiments showed that this low exposure frequency caused no detectable apoptosis due to the light per se. At the end of the experiment, the animals were euthanized, and ethanol (70%) was applied to the tissue to achieve 100% PI-labeling of all cell nuclei in the mesentery as reference.

Other DEX-treated groups were treated with two 180 μg/kg boluses of eptifibatide 10 minutes apart right before the administration of DEX. Monastral blue was injected 30 minutes before the end of the experiment to localize sites of increased capillary vascular permeability.

In other DEX-treated groups, fucoidin or anti L-selectin antibody (Lam1-116) were administered intravenously right before the administration of DEX.

3.2.6 Image and Statistical Analysis

The video images were analyzed off line. At each time point, a count was made of the number of PI-positive cells in a 140μm ? 100μm area in which a single capillary was centrally located. The measurements from each region in the mesentery, each time points, and each treatment group were averaged and presented as mean ± standard derivation.

Comparisons of mean values between animal groups were carried out by ANOVA. Chi-square test was used to test the difference between observed frequencies among animal groups. $P < 0.05$ was considered statistically significant.

3.3 Sub-Aim 1a. To Analyze the Correlation between Incidence of Stasis and Capillary Endothelial Cell Density

3.3.1 Arterial Blood Pressure

In this study, the mean blood pressures in the WKY rats and SHRs were 113 ± 4 and 164 ± 6 mmHg, respectively ($p < 0.05$). These values were maintained throughout the experiments.

3.3.2 Intravital Microscopic Observations

Typical capillaries in this study were about 8 to 10 μm in diameter with a single file of erythrocytes and regular passage of leukocytes and platelets. Individually blood cells became clearly visible at reduced blood velocity. In the mesentery parenchyma mast cells, fibroblasts, mesenchymal cells, and networks of collagen bundles were visible at the optical resolution employed in the current experiments. At the time the mesentery was exposed, there were no or very few PI-positive endothelial cells (< 1%) and normal blood flow in all capillaries (Figure 3.3.2 and Figure 4.4.1A and B at 10 min).

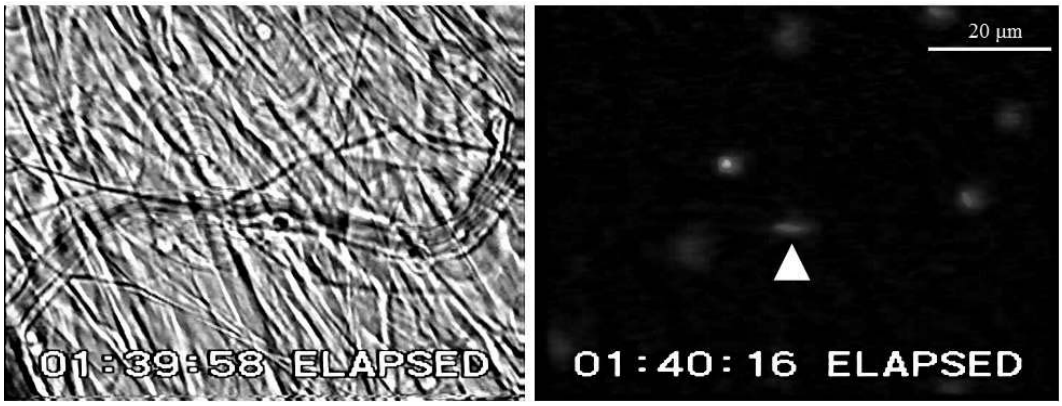

Figure 3.3.2 Bright field (left) and fluorescent micrograph for detection of blood flow stasis, cell morphology, cellular interactions, and apoptosis in capillary microvessels and in the adjacent tissue parenchyma. Fluorescent field images show nuclei of propidium (PI)-positive cells and their morphology. The images were recorded in a capillary of the DEX-treated WKY rat.

3.3.3 The SHR Has Fewer Capillary Endothelial Cells than the WKY Rat and Wistar Rat

The cell density in the mesentery before start of the experiment may influence measurements of cell apoptosis. Therefore, we determined the density of endothelial cells along the length of the capillaries after labeling of all cell nuclei at the end of the experiments. There was no difference in the total number cells (per 10,000-μm2 mesentery area) between strains (SHR 41.1 ± 2.7, WKY 44.3 ± 4.8, and Wistar 42.5 ± 6.1, Figure 3.3.3A). However, the number of capillary endothelial cells (per 100 μm capillary length) was lower in the SHR than the WKY and Wistar rat (2.4 ± 0.4 vs. 3.7 ± 0.6 and 3.6 ± 0.3, $p < 0.05$, Figure 3.3.3B). Along the SHR capillary wall there were frequently longer distances between adjacent endothelial nuclei (Figure 3.3.3C).

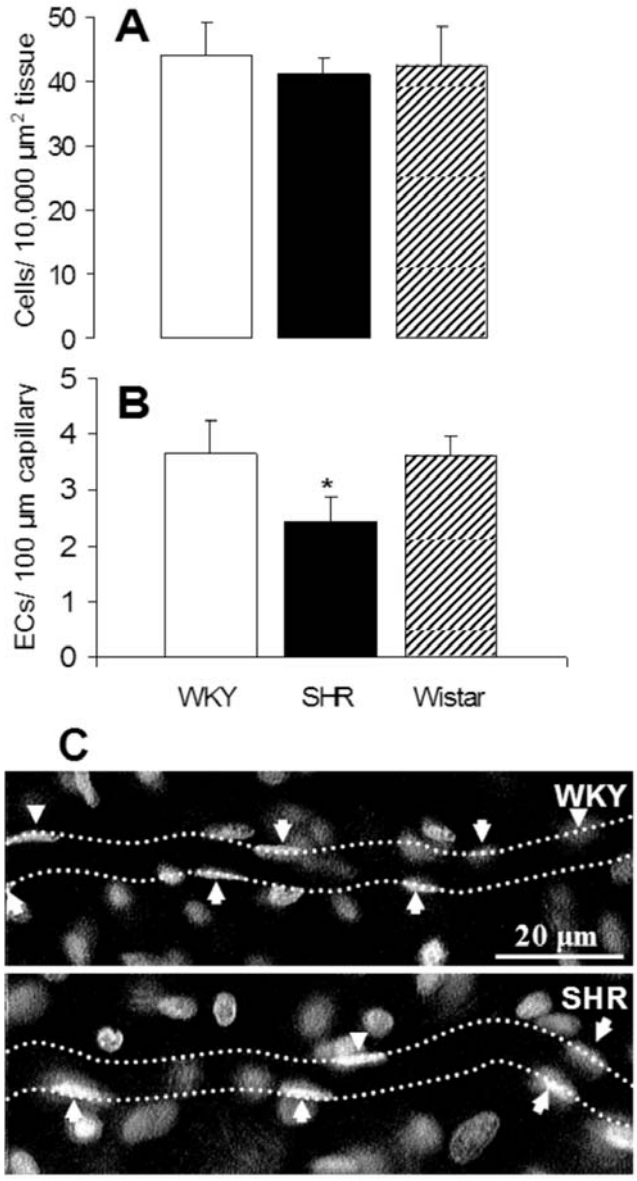

Figure 3.3.3 Preexisting elevated level of apoptosis in SHR. (A) Number of parenchymal cells per mesentery tissue area; (B) number of endothelial cells (EC) per unit length of capillary (with diameter less than about 10 µm), and (C) fluorescent images of cell nuclei (EC nuclei are indicated with a white arrow head, and the guide lines represent the capillary endothelium.) labeled with PI by superfusion with 70% ethyl alcohol to delineate all cell nuclei present in the observation field. Compared to normotensive rats (WKY n = 12 and Wistar n = 11), the SHR (n = 12) has the same number of mesenteric parenchymal cells (A), but fewer endothelial cells (B and C).

3.3.4 SHR Has Higher Incidence of Stasis

In 3 hours observation, the SHR has higher incidence of stasis, but it is not statistical difference (Figure 3.3.4)

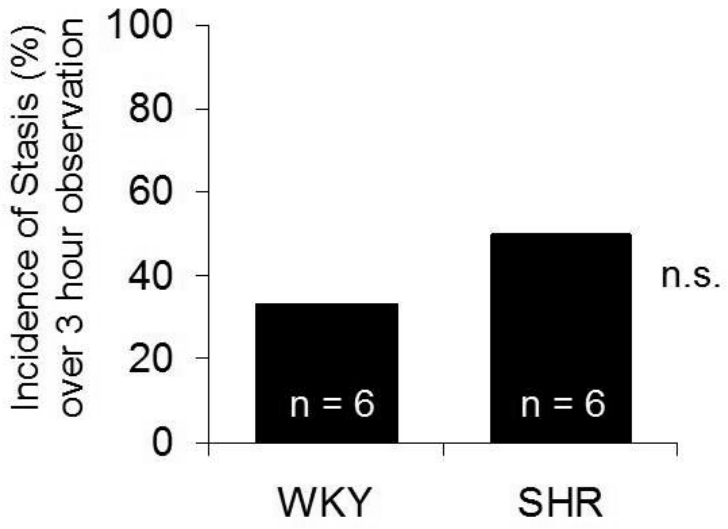

Figure 3.3.4 In 3 hours observation, the SHR has higher incidence of stasis, but it is not statistical difference

3.3.5 Capillaries with Fewer Endothelial Cells Have Higher Incidence of Stasis

Considering both number of capillary endothelial cells and permanent capillary stasis, both SHR and WKY show: the fewer endothelial cells the capillaries have, the higher incidence of stasis. Without the DEX treatment, the incidence of capillary stasis in the WKY and the SHR groups was inversely related to the number of endothelial cells per unit length of capillary (Figure 3.3.5).

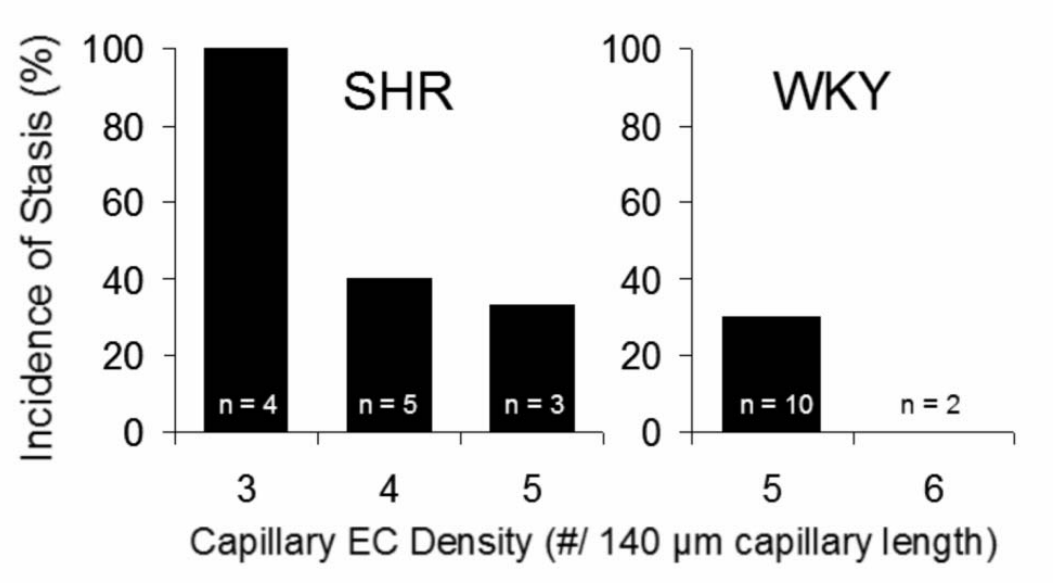

Figure 3.3.5 The incidence of capillary stasis in the WKY and the SHR groups was inversely dependent on the number of endothelial cells per unit length of capillary.

3.4 Sub-Aim 1b. To Analyze the Correlation between Stasis and Endothelial Cell Death

3.4.1 Arterial Blood Pressure

In this study, intraperitoneal injection and superfusion with DEX did not significantly change systemic blood pressure. The mean blood pressures in the WKY rats and SHRs were 113 ± 4 and 164 ± 6 mmHg, respectively ($p < 0.05$). These values were maintained throughout the experiments.

3.4.2 Glucocorticoid Induces Permanent Capillary Stasis

All animals in the WKY+DEX group (n = 6) developed permanent capillary stasis on average after 128 minutes observation of the tissue (248 minutes exposure to DEX). In contrast, only two animals in the control WKY group (n = 6) developed permanent capillary stasis after about 150 minutes observation. The incidence of permanent stasis in the WKY+DEX group was significantly higher than its control WKY group ($p = 0.014$). In contrast, there was no significant difference in the incidence of permanent capillary stasis between the hypertensive groups (5 out of 6 SHR+DEX vs. 3 out of 6 SHR), and between hypertensive groups and their normotensive counterparts (SHR+DEX vs. WKY+DEX, and SHR vs. WKY) (Figure 3.4.2C). The length of the time interval between the appearance of the first PI-positive endothelial cell and permanent stasis in a capillary was about 70 min and not significantly different between the groups. With dexamethasone treatment, in 3 hours

observation, the incidence of stasis increase significantly to 83% in SHR and 100% in WKY. We did not see any difference between strains

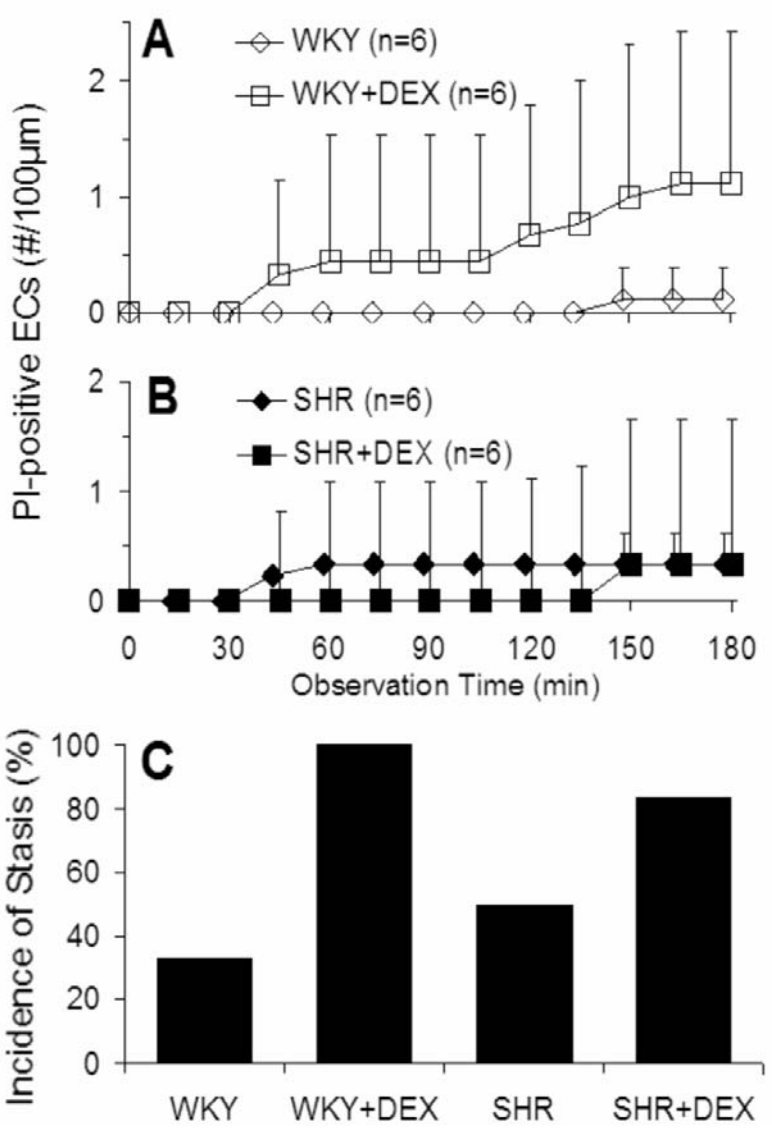

Figure 3.4.2 Endothelial cell death and capillary stasis induced by glucocorticoid (dexamethasone, DEX). In DEX-treated WKY, the faction of endothelial cell death and the incidence of capillary stasis increased significantly (A and C). In the SHR, the high level of preexisting endothelial apoptosis was not further increased by DEX application over a period of five hours.

3.4.3 Glucocorticoid Induces Apoptosis in WKY Endothelial Cells

In each group, the number of PI-positive cells increased in time. The majority of these PI-positive cells were located in the tissue parenchyma, with involvement of relatively few endothelial cells. In all animal groups, the spatial distribution of PI-positive cells in the mesentery was random. There was no difference in the density of PI-positive cells among any of tissue intervals from the vessel. In fact, in the WKY control group, almost no PI-positive endothelial cells were detected during the course of the experiment. Throughout the course of the experiment, the WKY+DEX group developed significantly higher levels of endothelial cell death than the WKY control group. The SHR group had an insignificantly higher incidence of endothelial cell death than the WKY group. We detected no difference in endothelial cell death between the SHR groups (Figure 3.4.2A and B).

Dexamethasone also induces endothelial cell death in WKY, but the difference in 3 hours observation is not statistically significant.

We do not see any change in SHR.

3.4.4 Capillary Stasis Leads to Progression of Endothelial Apoptosis

After complete capillary stasis, all endothelial cells along the vessel wall became apoptotic within a few hours. The rate at which endothelial apoptosis was progressing along a capillary after permanent stasis was faster than before stasis (The last images of Figure 4.4.1A and B).

Graph number of death ECs before and after stasis (Figure 3.4.4), we realize that:

1. Level of endothelial cell death increases after the stasis
2. Stasis happens at capillary with more endothelial cell death (late stasis in SHR+/-DEX and WKY)

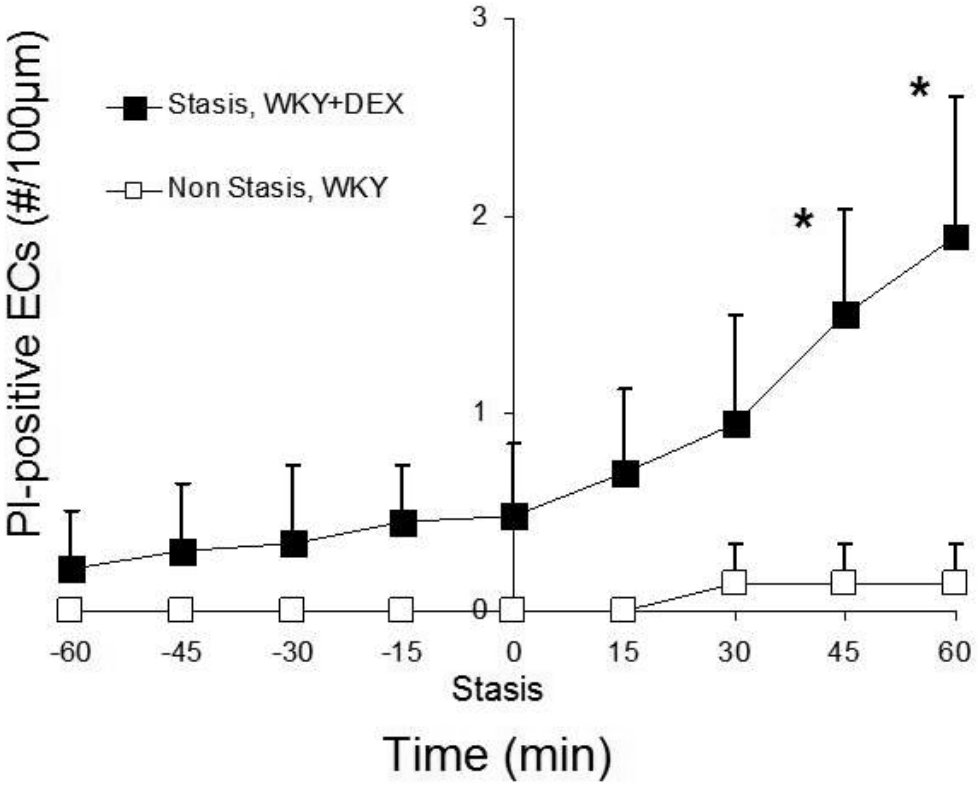

Figure 3.4.4 Rate of cell death before and after capillary stasis in WKY. After complete capillary stasis, all endothelial cells along the vessel wall became apoptotic within a few hours. The rate at which endothelial apoptosis was progressing along a capillary after permanent stasis was faster than before stasis. The graph of non-stasis WKY is used as a control rate.

3.5 Summary and Discussion

The SHR has fewer capillary endothelial cells and higher incidence of stasis

Capillaries with fewer endothelial cells have higher incidence of stasis

Glucocorticoids, known to be responsible for the development of hypertension, induces stasis and endothelial cell death in WKY

The capillary stasis leads to progression of endothelial apoptosis, the first step of structural rarefaction.

A distinction has been made between functional rarefaction, when microvessels are filled with plasma but not perfused with blood cells, and structural rarefaction, when vessels are actually no longer present. It has been proposed that the earlier functional rarefaction may lead eventually into structural rarefaction (34). Such a scenario may be possible in arterioles under vasoconstriction, but has not been demonstrated with a direct in-vivo approach.

After development of stasis in a capillary, we find within a few hours spreading of endothelial cell death along the length of the capillary segment that is not perfused but not in adjacent vessels or bifurcations that are still perfused. Furthermore, the lower endothelial cell counts (per capillary length) in the SHR agrees with Gobé's hypothesis that gradual sporadic apoptosis of capillary endothelial cells could lead to fewer endothelial cells exposing denuded basement membrane (14). Long-term exposure to DEX does lead to capillary rarefaction and reduces organ weight (50). Since in many organs the capillaries and parenchymal cells form an integrated

functional unit, the loss of capillaries is likely to cause parenchymal cell dysfunction, a potentially important pathophysiological process that requires further investigation under in-vivo conditions.

To study how stasis happens, I go to specific aim 2

3.6 Acknowledgements

Chapter 3, in part, has been submitted for publication of the material as it appears in An in-vivo Analysis of Capillary Stasis and Endothelial Apoptosis in a Model of Hypertension, 2007, Tran, Edward D., Schmid-Schönbein, Geert W., Microcirculation. The dissertation author was the primary investigator and author of this paper.

4 Specific Aim 2. To Determine the cellular events leading to capillary stasis

Hypothesis: apoptosis of endothelial cell leads to stasis

The second specific aim is to determine the cellular events leading to capillary stasis, with the following sub-aims:

Sub-Aim 2a. To identify apoptotic endothelial cells and endothelial permeability

Sub-Aim 2b. To identify the role of leukocytes and platelets leading to stasis

Sub-Aim 2c. To block the formation of capillary stasis with blockers to membrane adhesion molecules

4.1 Introduction:

4.1.1 Apoptotic Endothelial Cells Become Proadhesive

As showed in introduction, endothelial cells undergoing apoptosis have apoptotic bodies protruding into the vessel lumen (15). Apoptotic endothelial cells become proadhesive for nonactivated platelets (7). The basement membrane breakdown with collagen fragments was found around apoptotic endothelial cells (15).

4.1.2 Introduction: Platelets and Endothelial Cell Interaction with Leukocytes

Many studies have showed the important role of adhesion molecules on platelets and leukocyte to adhere to endothelium. Thrombin-activated platelets adhered to resting-endothelial cells at dynamic flow conditions through glycoprotein IIb/IIIa (GP IIb/IIIa) on the platelets (32). Adhesion of neutrophils to platelets depends on P-

selectin exposed on the platelets (25). L-selectin on leukocyte interacts with CD34 on endothelial cells (28).

4.2 Materials and Methods

Similar materials and methods were described above in specific aim 1 (see 3.2 Materials and Methods).

4.2.1 Annexin V-propidium iodide (PI) double stain

Apoptotic endothelial cells were indicated by annexin V and propidium iodide (PI) (see 1.4.4B and Figure 4.2.1)

Live endothelial cells showed no or only weak annexin V staining of the cellular membrane, while early-stage apoptotic endothelial cells showed a significantly higher degree of surface labeling. Late-stage apoptotic endothelial cells showed both membrane staining by annexin V and nuclear staining by the propidium iodide.

Phosphatidylserine molecules locate on inner layer of plasma membrane of live cells.

In early stage of apoptosis, the molecules flip to outer layer, and are detected with Annexin V. This happen long before the cells are detectable with PI

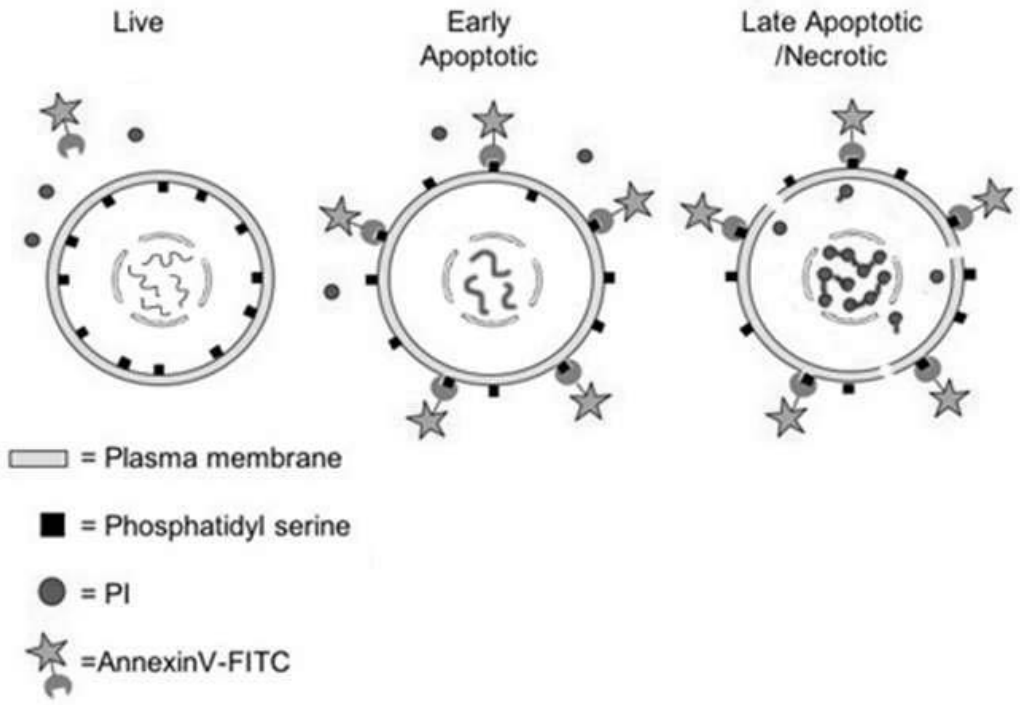

Figure 4.2.1 Live endothelial cells showed no or only weak annexin V staining of the cellular membrane, while early-stage apoptotic endothelial cells showed a significantly higher degree of surface labeling. Late-stage apoptotic endothelial cells showed both membrane staining by annexin V and nuclear staining by the propidium iodide.

4.2.2 Blockers of Membrane Adhesion Molecules

See 3.2.3

4.3 Sub-Aim 2a. To Identify Apoptotic Endothelial Cells and Endothelial Permeability

4.3.1 Endothelial Cell Apoptosis

Live endothelial cells showed no or only weak annexin V staining of the cellular membrane, while early-stage apoptotic endothelial cells showed a significantly higher degree of surface labeling. Late-stage apoptotic endothelial cells showed both membrane staining by annexin V and nuclear staining by the propidium iodide (Figure 4.3.1)

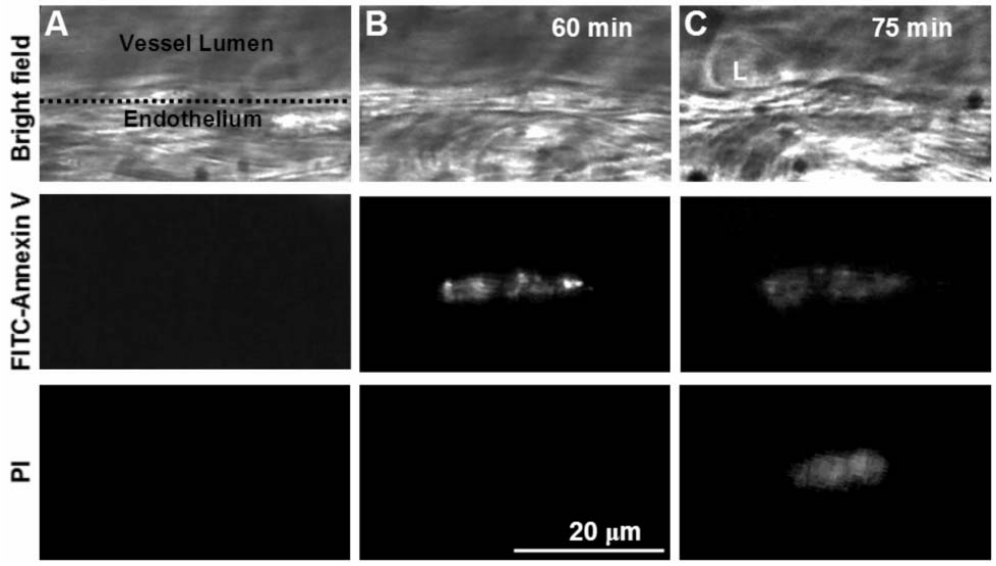

Figure 4.3.1 Endothelial cell death was indicated with annexin V/propidium iodide double stain. Live cells (A) did not show annexin V staining of the cellular membrane, while early-stage apoptotic cells (B) showed a significantly higher degree of surface labeling. Late-stage apoptotic cells (C) showed both membrane staining by annexin V and nuclear staining by the propidium iodide, and a leukocyte (L) bound to the late-stage apoptotic endothelial cell. The images were recorded in a capillary of the DEX-treated SHR.

4.3.2 Endothelial Permeability Increases Coincide with the Position of Death Endothelial Cells

Increased vascular permeability was associated with apoptotic endothelium. In DEX-treated animals, the sites of increased vascular permeability, indicated by Monastral blue leak, co-incited with the location of apoptotic endothelial cell (Figure 4.3.2).

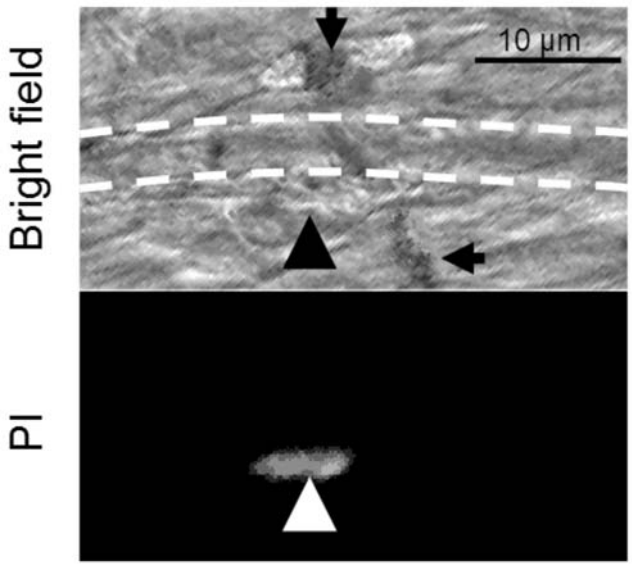

Figure 4.3.2 In top panel, bright filed image of a capillary site with increased vascular permeability as indicated by Monastral blue leak (small arrows), located near an apoptotic endothelial cell (large arrow) as shown by the PI-labeling (fluorescent image in lower panel). The guide lines represent the capillary endothelium. The images were recorded in a DEX-treated WKY rat.

4.4 Sub-Aim 2b. To identify the role of leukocytes and platelets leading to stasis

Typical capillaries in this study were about 8 to 10 μm in diameter with a single file of erythrocytes and regular passage of leukocytes and platelets. Individually blood cells became clearly visible at reduced blood velocity. In the mesentery parenchyma mast cells, fibroblasts, mesenchymal cells, and networks of collagen bundles were visible at the optical resolution employed in the current experiments. At the time the mesentery was exposed, there were no or very few PI-positive endothelial cells (<1%) and normal blood flow in all capillaries (Figure 3.3.2 and Figure 4.4.1A and B at 10 min).

4.4.1 Adhesion of Leukocytes to Apoptotic Endothelial Cell Leads to Capillary Stasis.

Blood flow in individual capillaries may accelerate, decelerate, stop, or even temporarily reverse. In all animal groups, during flow, there was no unique velocity pattern associated with the progression of endothelial apoptosis. Without exception in all capillaries of all animal groups we saw attached platelets and temporary stasis before we could see permanent stasis. In many cases, leukocytes adhered temporarily to the capillary endothelium, caused temporary capillary stasis, but then detached again and allowed restoration of blood flow.

In contrast, when one or more leukocytes adhered firmly to the capillary endothelium with platelets attached to its membrane, erythrocyte aggregates formed

upstream and downstream of the leukocytes, and the capillary developed permanent stasis. Stasis was observed in all (out of a total of 24) cases only if a leukocyte was present; we observed no case in which platelet aggregates grew to a size that was sufficient to completely obstruct the lumen of a capillary. Endothelial cells at the site of a capillary with an entrapped leukocyte became PI-positive before or soon after (within about 15 minutes) the instant when the capillary became permanently obstructed (Figure 4.4.1A and 4.4.1B, respectively). When the experiment was repeated with annexin V, the endothelial cells where platelets and leukocytes adhered firmly to were in either late stage of apoptosis (annexin V-positive and PI-positive) or early-stage of apoptosis (annexin V-positive and PI-negative).

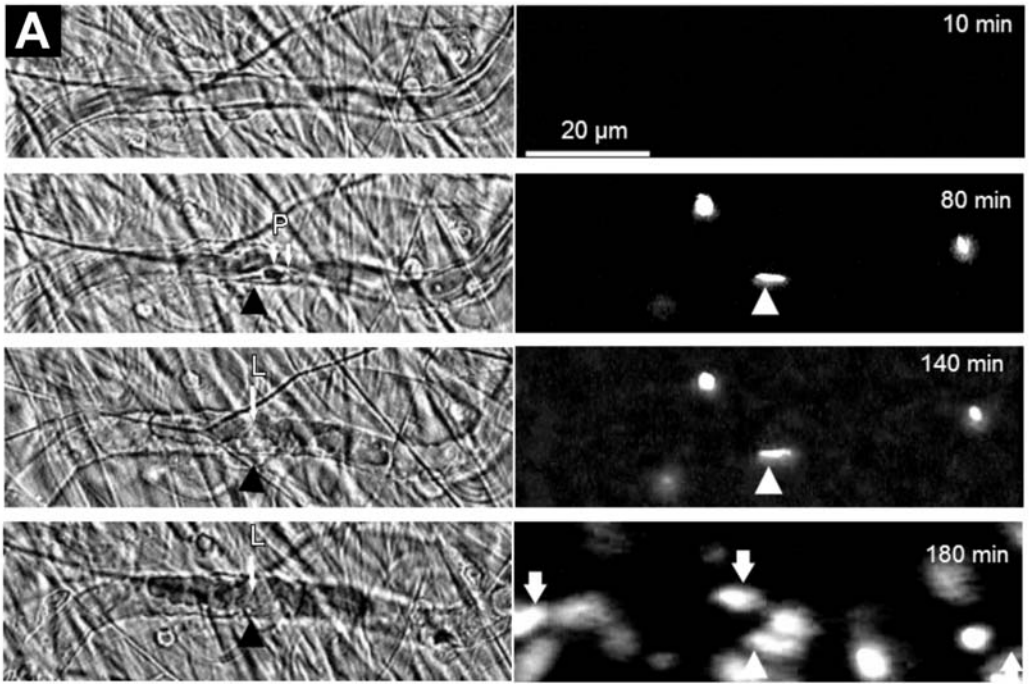

Figure 4.4.1 *In-vivo* bright field micrographs of capillary flow (left column) and apoptosis (right column) as detected by labeling with propidium iodide (PI) **Series A:** Capillary flow was initially stopped by a single leukocyte followed by red blood cell aggregation and thrombus formation with capillary endothelial apoptosis. At 10 min, normal flow was seen in bright field and no cell death in the fluorescence field. At 80 min, an endothelial cell (EC) died (black arrow); platelets (P) and red blood cells adhered on its surface. At 140 min, a leukocyte (L) stopped, followed by thrombus formation. At 180 min, most ECs along the capillary became PI-positive (white arrows, right images). The images were recorded in capillaries of the DEX-treated WKY rat. **Series B:** Example of leukocyte attachment in a capillary without prior PI-positive endothelial cell (at 170 min). Ten minutes after leukocyte entrapment, the endothelial cells became PI-positive (white arrows, right images). In this case, we observed leukocyte attachment to the endothelium before the first PI-positive capillary endothelial cell. The images were recorded in capillaries of the WKY rat.

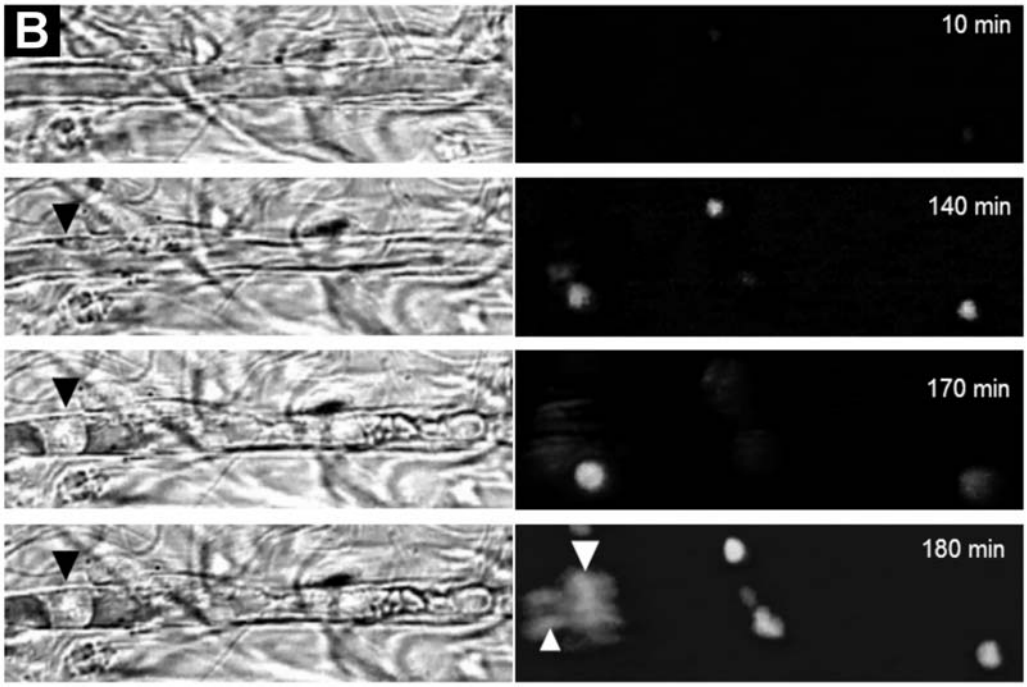

Figure 4.4.1 (continued)

4.4.2 Platelet Requirement for Permanent Capillary Stasis

Normal capillary endothelium without stasis was smooth and formed a thin layer without any indication for platelet or leukocyte attachment. Magnified images showed that capillary stasis occurred at endothelial cells that had cytoplasmic projections into the lumen and attachment of immobilized platelets. Without exception we saw platelet attachment and temporary stasis before permanent capillary stasis. Later, a leukocyte was trapped at the same location and an aggregate with several platelets and erythrocytes was formed (Figure 4.4.2) that lead to complete obstruction. During this period of time we saw not evidence for actual collapse of the capillary lumen.

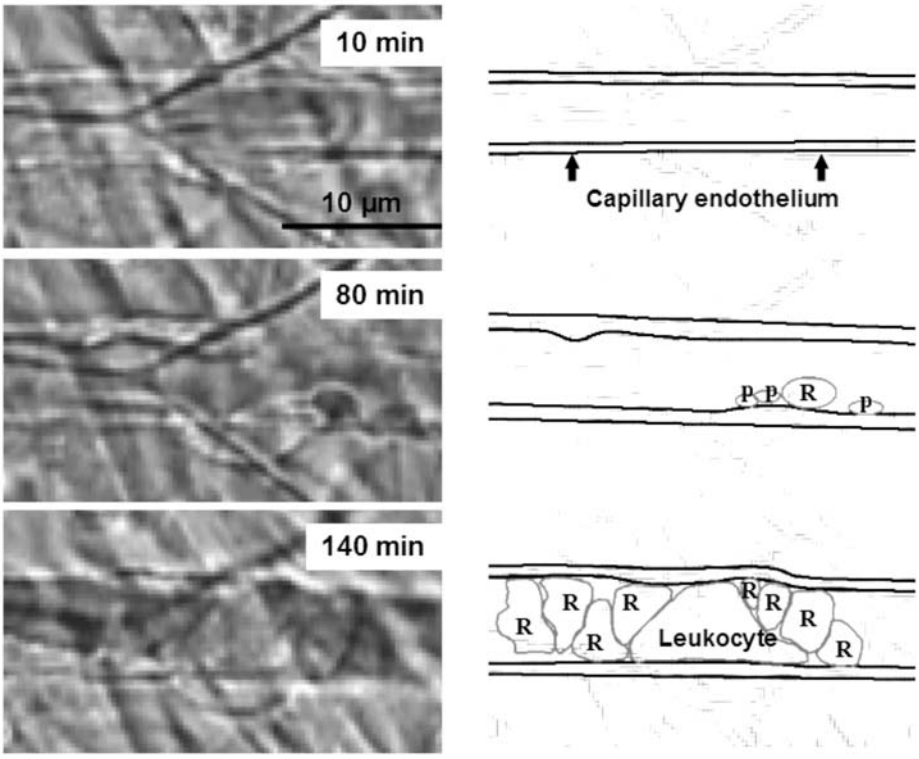

Figure 4.4.2 Bright field image (left) and line tracing (right) of capillary stasis at a site where endothelial cells protruded into the capillary lumen. The tracing was generated by means of the full video record. A normal capillary endothelium was shown at 10 min (top panels). At 80 min (middle panels), the capillary endothelium became thicker, endothelial cells projected into the lumen, and there was attachment of platelets (p) and red blood cells (R). At 140 min (lower panels), a leukocyte was trapped at the endothelial cell, and a cell thrombus was formed.

4.5 Sub-Aim 2c. To Block the Formation of Capillary Stasis with Blockers To Membrane Adhesion Molecules

In DEX-treated WKY rats (n = 5) and DEX-treated SHRs (n = 5), glycoprotein IIb/IIIa blockade with eptifibatide inhibited platelet attachment on endothelium and platelet aggregation, and it completely prevented permanent capillary stasis under the current experimental conditions ($p < 0.01$, Figure 4.5A). However, eptifibatide did not prevent temporary stasis and Monastral blue leak.

Monoclonal antibody against L-selectin on leukocyte inhibited attachment of leukocyte to platelets and endothelium also completely prevented permanent capillary stasis under the current experimental conditions ($p < 0.01$, Figure 4.5A). Furthermore, it could reverse a few-hour stasis in capillaries as well as arterioles and venules (Figure 4.5B).

Fucoidin attenuated leukocyte rolling and lowered incident of permanent capillary stasis in DEX-treated WKY rats (n = 4) and DEX-treated SHRs (n = 4) to the control level ($p < 0.05$, Figure 4.5A), but did not prevent permanent capillary stasis completely.

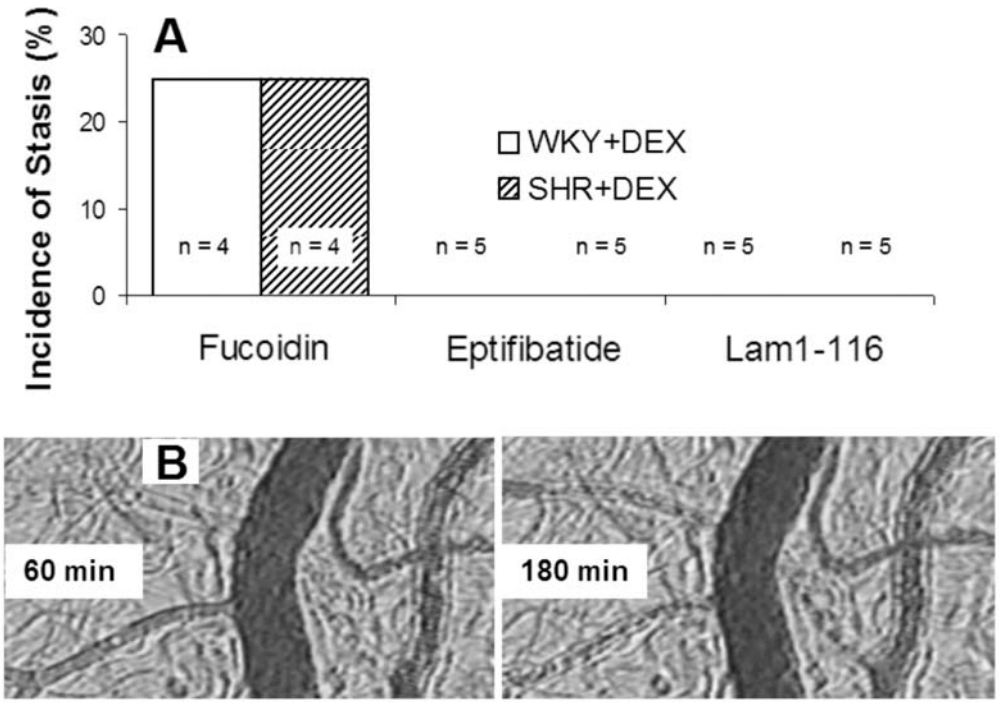

Figure 4.5 In DEX-treated WKY rats and DEX-treated SHRs, eptifibatide (glycoprotein IIb/IIIa blockade) and Lam1-116 (monoclonal antibody against L-selectin) completely prevented permanent capillary stasis. Fucoidin lowered incident of permanent capillary stasis to the control level. All changes were significant (A). Lam1-116 could reverse a two-hour stasis in capillaries as well as arterioles and venules (B). Stasis was observed in all vessels at 60 min, and then blood cells flowed through all the vessels at 180 min. The images were recorded in a DEX-treated SHR.

4.6 Summary

Capillary leakage coincide with the position of apoptotic endothelial cells

The cellular events of capillary stasis

• Platelets adhere to apoptotic endothelial cells

• Endothelial apoptosis and platelet adhesion mediate adhesion of leukocytes to the endothelium, leading to capillary stasis

• Initial capillary stasis is rapidly followed by red blood cell aggregation and thrombus formation

Blockers of membrane adhesion molecules (GP IIb/IIIa and L-selectin) prevent capillary stasis over 5-hour period.

4.7 Acknowledgements

Chapter 4, in part, has been submitted for publication of the material as it appears in An in-vivo Analysis of Capillary Stasis and Endothelial Apoptosis in a Model of Hypertension, 2007, Tran, Edward D., Schmid-Schönbein, Geert W., Microcirculation. The dissertation author was the primary investigator and author of this paper.

5 Discussion

5.1 A Cellular Process for Capillary Stasis

The current in-vivo observations bring to light a mechanism that may be involved in capillary rarefaction as encountered in arterial hypertension. Stasis in these narrow vessels is initiated at individual endothelial cells without blood pressure reduction in central arteries. Stasis is caused by entrapment of individual leukocytes (mostly granulocytes), followed by endothelial apoptosis and formation of red cell aggregates. This in turn is likely followed by fibrin formation, since the entire length of the capillary from the upstream to the downstream bifurcation ceases any blood cell motion. Capillaries may be subject to temporary stasis, a process that is also caused by leukocytes (5, 41), until it leads within hours to irreversible stasis. Irreversible stasis can be seen before the entire set of endothelial cells that make up the capillary becomes PI-positive.

5.2 Annexin V/PI Double Stain Indicates That Annexin V Positive Cells Need at Least 15 Minutes to Be Detectable with PI

As show in introduction of apoptosis, the early stage of apoptosis happens long before the late stage of apoptosis, and my result indicates that annexin V positive cells need at least 15 minutes to be detectable with propidium iodide.

The development of capillary stasis requires apoptotic endothelial cell in early stage or in late stage of apoptosis.

5.3 Apoptosis of Capillary Endothelial Cells in Hypertension

After dexamethasone treatment, the normotensive WKY strain developed higher levels of cell death and permanent capillary stasis than the SHR strain. This observation is in contrast to increasing evidence which indicates that the SHRs' blood vessels and cells respond to glucocorticoids more pronounced than the WKYs' (12, 29). Adrenalectomy leads to normalization of the SHR blood pressure , while exposure to equal concentration of DEX causes higher blood pressure and apoptotic levels in the SHR (11, 17, 29, 30, 45) than the normotensive WKY controls. In the mesentery of the rat, we find enhanced apoptosis both in capillary endothelial cells as well as in parenchymal cells (29). DEX may serve to enhance the turnover rate of the SHR endothelial or parenchymal cells (18), or the cell turn-over rate may be lowered in normotensive rats. Exposure of the SHRs to exogenous DEX did not lead to enhanced apoptosis compared with normotensive controls. It is uncertain at the moment why this is the case, but it is possible that (1) the cell survival in SHR may be reduced by endogenous glucocorticoid and resist apoptosis, or (2) the SHR cells may be on average in a different stage of their cell cycle. Untreated SHR and DEX-treated SHR show almost identical levels of capillary stasis and cell death in the mesentery (Figure 3.4.2A and B).

5.4 The Important Role of the Lectin Domain of L-Selectin in Permanent Stasis

Our experiments with fucoidin and anti L-selectin antibody indicate a decisive role of the lectin domain of L-selectin over the mechanism responsible for permanent stasis in capillaries. In the current experiment, the antibody did not block the

leukocyte rolling completely but it was able to prevent the capillary stasis. Furthermore, the antibody could reverse stasis in capillaries as well as arterioles and venules (Figure 4.5B). In normotensive rats after occlusion of microvessels (with a micropipette), leukocytes project pseudopods while in free suspension or attached to the endothelium. When flow is restored, pseudopods on adhering leukocytes retract and the cells begin to roll and detach from the endothelium (33). In the current control experiments, the flow in rat mesentery microvessels was never restored by itself after an hour occlusion. The pseudopods of leukocytes and attachment of leukocytes to the endothelium may increase the capillary resistance. Besides being an adhesion molecule, L-selectin activates the Ras pathway via the tyrosine kinase p56 lck (8). Anti L-selectin antibody might inhibit leukocyte activation, and then help to restore the flow in microvessels.

5.5 Endothelial Alteration and Platelet Adhesion Lead to Attachment of Leukocytes to the Capillary Endothelium and Capillary Stasis

5.5.1 Apoptotic Vascular Endothelial Cells Become Procoagulant

The integrity of the endothelium is crucial for the maintenance of the antithrombotic state. Perturbation or injury of endothelial cells leading to exposure of tissue factor and extracellular matrix are well known to induce the coagulation process.

Our observations clearly indicate that platelets are attached to the endothelial cell where permanent stasis occurs. We observed no permanent adhesion of leukocytes to capillary endothelium in the absence of platelets. The glycoprotein

IIb/IIIa blocker, eptifibatide, blocked attachment of platelets to the endothelium and prevented permanent capillary stasis. Endothelial cells undergoing apoptosis become proadhesive for circulating platelets (7). Even a small number of platelets that adhere to morphologically intact endothelium or activated endothelial cells have the ability to capture flowing neutrophils in capillaries and facilitate their immobilization at the vessel wall and so promote inflammatory and thrombotic intercellular interactions (25, 31, 32, 38, 55). Over the time period of the current experiments we saw no evidence for an actual closure of the capillary lumen caused by a shape change of the endothelial cells (e.g. swelling of endothelial cells, cytoplasmic pseudopod formation and alike).

A surprising observation in the current experiments is the fact that glucocorticoids may be involved in the formation of capillary stasis. Glucocorticoids are recognized to block adhesion of leukocytes to postcapillary endothelium (10, 50). Glucocorticoids also have a significant control over arteriolar tone (49), free radical production in arterioles and venules (47). In glucocorticoid-dependent forms of hypertension, like the SHR, this situation is accompanied by suppression of leukocyte adhesion to endothelium of venules (4, 46, 48) due to suppression of P-selectin expression (44).

In contrast to larger vessels (16) or post-capillary venules (23, 51), less is know about the expression of adhesion molecules in true single-file capillaries even though these vessels make up the great majority in the circulation. Immunolabeling in the mesentery indicates a sharp transition of ICAM-1 expression from almost undetectable levels in capillaries to enhanced expression of ICAM-1 at the site where capillaries

join to form the first postcapillary venules (23). The distribution appears to be different in other organs (3). The distribution of selectins in capillaries is largely unexplored. Leukocyte adhesion to capillary endothelium is regularly observed after application of inflammatory mediators or after ischemia/reperfusion, but both of these conditions were not present in the current study.

When venular endothelial cells become activated, they express and activate several adhesion molecules on their surface (e.g. P-selectin) to facilitate leukocyte adhesion in conjunction with expression of von Willebrand factor (52). Under physiological blood flow, leukocytes stop temporarily and roll before they firmly adhere. During membrane contact and rolling on postcapillary venules the cells may activate each other (24, 27).

In capillary endothelium the membrane adhesion molecules are less explored. In the current study, the presence of PI-positive endothelial cells in capillaries leads to leukocyte entrapment at these sites, a process that is facilitated by platelets (32). We also observed leukocyte entrapment at capillary endothelial cell whose cell body protruded into the lumen of the capillary with immobilized platelets, but without indication that the cell had become PI-positive. This evidence indicates that endothelial apoptosis is not the precipitating event in capillary stasis and instead it is the attachment of platelets to the capillary wall. Since the platelet attachment can be prevented by blockade of its integrin IIb/IIIa, a molecule that attaches readily to the basement membrane proteins (1, 36), the evidence suggest that one of the earliest events may be opening of the cell junctions with leakage of monastral blue between endothelial cells and exposure of the underlying basement membrane. The endothelial

cell became PI-positive within minutes after development of permanent capillary stasis. During the entire process the leukocytes in obstructed capillaries exhibit little passive deformation likely due to their high cytoplasmic stiffness (40).

5.5.2 Apoptotic Endothelium is Exposure of Collagen Fibers and Increase Permeability

Because, as a general feature of apoptosis, cells rapidly retract, round up, and subsequently lose their intercellular contacts,56 it was assumed that apoptotic HUVECs may expose substantial areas of the highly procoagulant subendothelial matrix. Indeed, even without cell detachment, HUVECs increased collagen exposure from 5% to 30% within the first 6 hours of cell death, thereby inducing dramatic enhancement of thrombin formation in recalcified plasma. This procoagulant effect is analogous to a previous study showing that the anticoagulant effect of endothelial cells in whole blood or plasma is overcome by exposure of collagen by about 5% to 10% in dependence on the presence of platelets. Because retraction of endothelial cells upon exposure to inflammatory cytokines has been shown to involve protein kinase C,57 it is possible that staurosporine might influence the retraction process. However, staurosporine rather inhibits cell retraction or induces cell spreading. Thus, it is likely that an area of apoptotic endothelial cells in vivo may lead to significant exposure of subendothelial matrix. The significance of enhanced PS exposure and loss of anticoagulant molecules in plasma seems to be reflected in the observation that floating apoptotic HUVECs were also found to increase thrombin formation in plasma. Using a modified Russel viper venom test, a recent study has similarly shown

that apoptotic leukocytic and endothelial cells exert a procoagulant effect in plasma.60 Thus, it is tempting to speculate that detached endothelial cells may lose immediately their anticoagulant properties and circulate as procoagulant bodies in a platelet-like manner.

Small molecule monastral blue can cross the damage of tight junctions in the apoptotic endothelium.

5.6 Capillary Stasis Leads To Progression of Endothelial Apoptosis and Microvascular Rarefaction

A distinction has been made between functional rarefaction, when microvessels are filled with plasma but not perfused with blood cells, and structural rarefaction, when vessels are actually no longer present. It has been proposed that the earlier functional rarefaction may lead eventually into structural rarefaction (37). Such a scenario may be possible in arterioles under vasoconstriction, but has not been demonstrated with a direct in-vivo approach.

After development of stasis in a capillary, we find within a few hours spreading of endothelial cell death along the length of the capillary segment that is not perfused but not in adjacent vessels or bifurcations that are still perfused. Furthermore, the lower endothelial cell counts (per capillary length) in the SHR agrees with Gobé's hypothesis that gradual sporadic apoptosis of capillary endothelial cells could lead to fewer endothelial cells exposing denuded basement membrane (15). Long-term exposure to DEX does lead to capillary rarefaction and reduces organ weight (53). Since in many organs the capillaries and parenchymal cells form an integrated

functional unit, the loss of capillaries is likely to cause parenchymal cell dysfunction, a potentially important pathophysiological process that requires further investigation under in-vivo conditions.

5.7 Conclusion

Endothelial apoptosis and platelet adhesion lead to adhesion of leukocytes to the endothelium, which in turn causes capillary stasis

The capillary stasis leads to progression of endothelial apoptosis, the initial step in structural rarefaction

In hypertension, elevated level of apoptosis of endothelial cells in microcirculation plays an important part in the cellular mechanisms responsible for enhanced microvascular complications in hypertension.

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