CLIMACTERIC 2003;6:293–301

Progestins initiate adverse events of menopausal estrogen therapy

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Key words: HORMONE REPLACEMENT THERAPY, MENOPAUSE, ESTROGEN, PROGESTERONE, PROGESTIN, CARDIOVASCULAR DISEASE, THROMBOEMBOLISM, STROKE, DEMENTIA

ABSTRACT

Objective Recent reports, particularly the Women's Health Initiative, demonstrated that hormone therapy with combined estrogen plus progestin increased the incidence of heart attacks, stroke, blood clots, breast cancer and dementia in women over 65 years old. We investigated the role of synthetic progestins in initiating the adverse events associated with estrogen therapy.

Methods We used a fluorescence imaging technique, which allows video microscopic recordings of blood flow, blood vessel morphology and activities of various blood cells in a live animal. The acute peripheral and cerebrovascular responses were measured following intraperitoneal (5 or 10 mg) or intravenous (10 or 100 μg) administration of progesterone, synthetic progestins (medroxyprogesterone acetate and norethindrone) or estrogens (conjugated equine estrogens and 17β-estradiol).

Results In both peripheral and cerebral vasculature, synthetic progestins caused endothelial disruption, accumulation of monocytes in the vessel wall, platelet activation and clot formation, which are early events in atherosclerosis, inflammation and thrombosis. Natural progesterone or estrogens did not show such toxicity.

Conclusions The risks associated with combined estrogen plus progestin therapy may be a consequence of vascular actions of progestins. Using progestins with minimal vascular toxicity may lead to safer estrogen preparations for menopausal women.

INTRODUCTION

The medical, economic and human impact of conditions associated with the menopause – vasomotor symptoms, urogenital atrophy, osteoporosis, cardiovascular disease and dementia – are enormous[1,2]. Hormone replacement is used by about 38% of postmenopausal women in the USA, and estrogen preparations are one of the most widely prescribed medications[3]. A synthetic progesterone (progestin) is added to estrogen to protect against estrogen-induced endometrial hyperplasia and uterine cancer. Cardiovascular disease (CVD), including sudden death, is the leading cause of mortality in women. In addition, the incidence of Alzheimer's disease is two-fold higher in postmenopausal women when compared with men[2]. Several observational epidemiological studies have shown up to 50% lower rates of cardiovascular disease[4] and Alzheimer's disease[5,6] among postmenopausal women using estrogen therapy. However, a number of recent randomized clinical trials found no benefit of hormone replacement therapy (HRT) on cardiovascular disease[7–10] and Alzheimer's disease[11–13]. Furthermore, elevated levels of endogenous[14] or

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ORIGINAL ARTICLE

Received 14–07–03
Revised 17–09–03
Accepted 18–09–03

exogenous[15] estrogen were found to have no beneficial effect on cognitive function in elderly women. These discrepancies in outcomes of estrogen therapy were to be resolved by the first long-term, randomized, prospective, placebo-controlled trial, the Women's Health Initiative (WHI)[16].

One part of the WHI trial, the estrogen–progestin subgroup of the HRT arm, was terminated in May 2002 after 5.2 years of follow-up, when the number of adverse events reached a predetermined limit[16]. The WHI report showed higher numbers of invasive breast cancers, heart attacks, strokes and thrombotic events among the 8506 women assigned to the study medication (0.625 mg of conjugated equine estrogens plus 2.5 mg of medroxyprogesterone acetate per day), than among the 8102 women in the placebo group. Even though the absolute risk of harm to an individual woman was very small, it was recommended that estrogen–progestin therapy should not be used for the prevention of chronic diseases in postmenopausal women.

The Women's Health Initiative Memory Study (WHIMS)[17–19], an ancillary study to the WHI using combined estrogen plus progestin therapy, showed a two-fold increase in the risk of dementia in women aged 65 years and older. The increase was primarily due to higher rates of vascular-related dementia, and may have been due to a two-fold higher incidence of stroke[20] in the hormone-treated group. This observation also supports the view that vascular disease and silent brain infarcts may increase the risk for dementia[21].

The discordance between observational and clinical trials regarding the benefits and risks of hormone therapy may be the result of a number of factors such as advanced age of the subjects, occult disease[22], proinflammatory state[23], years past menopause, duration of hormone use, composition of hormone formulation, doses used, route of administration, formation of catechol estrogens and genetic or metabolic variation between subjects. In the majority of observational studies, unopposed estrogen was used, whereas clinical trials such as the Heart and Estrogen/progestin Replacement Study (HERS) and WHI used a combination of estrogen and progestin. The estrogen-only arm of the WHI is not plagued by a significant number of adverse events, and that segment of the study is ongoing. This has led to the suggestion that the progestin, particularly medroxyprogesterone acetate, may be the major factor contributing to the observed complications. Several investigators have also suggested that the addition of a progestin may antagonize the beneficial effects of estrogen through procoagulant and proinflammatory actions[19,20]. In the present study, we investigated the acute vascular, proinflammatory and thrombotic actions of progestins as a trigger for the adverse events associated with combined estrogen and progestin therapy.

METHODS

Animal subjects and surgical preparation

This study was approved by the institutional animal care and use committee. Female Sprague–Dawley rats (ovariectomized and non-ovariectomized) were anesthetized, and a femoral venous catheter was inserted and positioned with its tip 1–2 cm into the vena cava inferior as described previously[24,25]. The arterial blood pressure was measured with a Statham P23Db pressure transducer (AD Instruments, Colorado Springs, USA), connected to a cannula introduced into the aorta through the femoral artery. A heating pad was used to maintain body temperature, which was monitored using a rectal thermometer.

Peripheral vascular activity

The mesenteric blood vessels in live animals were studied as described before by Thomas and colleagues[24,25]. The mesentery is transparent, making it possible to observe blood flow and the interaction of leukocytes and platelets with the vascular wall in regular light microscopy. Fluorescein tagging of leukocytes and platelets with rhodamine 6G (Sigma Chemical Co., St. Louis, MO, USA) facilitates the observation of vascular activity using epi-illumination microscopy. The mesenteric field was video recorded prior to and following various treatments with an Optronics digital camera (DEI-750C) (C. Squared Corp., Tamarac, FL, USA).

Study medications

We compared the acute vascular actions of progesterone, progestins (medroxyprogesterone aceteate, MPA; norethindrone) and estrogens (conjugated equine estrogens, CEE; 17β-estradiol). For intraperitoneal administration, the hormones (5 or 10 mg) were dissolved in oil or carboxymethyl cellulose. For intravenous

administration, the compounds (10 or 100 μg) were dissolved in polyethylene glycol. Control animals received the corresponding solvents alone.

Cerebral vascular activity

The cranial window preparation used to investigate the cerebrovascular actions of progestins and estrogens is a modification of previous techniques[26]. The anesthetized rat was secured on the heating pad in a stereotactic head-device, in the prone position to facilitate preparation. The top of the skull was shaved, and a mid-line, longitudinal skin incision was made, exposing the parietal bone. With a high-speed drill, an oval 8 × 4-mm craniotomy was performed on one side of the skull mid-line. The dura matter on one side of the sagittal sinus was carefully excised. During this process and subsequently, the brain surface was irrigated with artificial cerebrospinal fluid. The leptomeningeal (pial) blood vessels were viewed and recorded during the entire duration of the experiment with a video microscopic recording system consisting of an Olympus BX30 epifluorescence microscope (C. Squared Corp., Tamarac, FL, USA)[26].

Leukocyte and platelet imaging

Polymorphonuclear leukocytes, monocytes, lymphocytes and platelets were labelled orange/yellow with 20 μl rhodamine 6G (200 μg/ml in saline), followed by another 10 μl 30 min later. The blood plasma was seen as positive green by using a single dose of fluorescein-isothiocyanate (FITC, 1 μg/100 g body weight). The number of marginating (sticking) leukocytes/monocytes and platelets was counted in a 1000-μm segment of the blood vessel. To qualify as 'marginating', leukocytes and platelets had to remain stationary for more than 10 s. The figures show representative images from four or more animals per group.

Data analysis

Morphological differences between control and treated animals were compared using previously described techniques[24–26].

RESULTS

Vascular actions of progestins (MPA and norethindrone)

In the cerebral (leptomeningeal) blood vessels, there was a rapid and intense response in the venules and capillaries (Figure 1). Rhodamine-tagged leukocytes and platelets became marginated, and adhered to the vessel walls. The leukocytes would occasionally transmigrate the venular walls, and the platelets would aggregate and adhere in venules, often resulting in hemostasis and the formation of small and large thrombi. Eventually, the endothelial lining of venules and capillaries became fluorescent, most readily noticed in the perinuclear region of the endothelial cells. In addition, the perivascular connective tissue space around large and small venules as well as capillaries displayed a diffuse fluorescence. The response in arterioles was minimal, and consisted of some leukocyte and platelet margination and occasional clot formation.

In the peripheral (mesenteric) blood vessels, the response was similar in arterioles and venules (Figure 2b, c and d). It consisted of margination and adherence of rhodamine-tagged leukocytes and platelets, leukocyte transmigration and platelet aggregation, clot formation, hemostasis and eventually thrombus formation. Gradually, the endothelial lining of arterioles, venules and capillaries became fluorescent. There was a diffuse fluorescence in the perivascular connective tissue space of all three types of microvessels, indicating vessel wall damage and breach of permeability barriers. To rule out the contribution of fluorescent lighting conditions in producing the vascular response, we also conducted these studies using normal visible light. Under light microscopic observation also (Figure 3c and d), there were characteristic features of endothelial damage and inflammation, including leukocyte margination and transmigration in arterioles and venules, platelet activation and mast cell degranulation. Intravenous administration of progestins produced a reaction in the venules within 10 min, whereas the subcutaneous administration initiated a response in about 30 min. In both cases, the reaction in arterioles was detected 30 min later.

Vascular actions of progesterone and estrogens

The responses to subcutaneous or intravenous administration of the natural progestational hormone progesterone were similar in the peripheral mesenteric microvasculature and the cerebral blood vessels. In either case, the appearance as well as the reactivity of arterioles, venules and capillaries was completely normal, and similar to that in control experiments in the absence of hormones (Figures 1b and 2a). Even

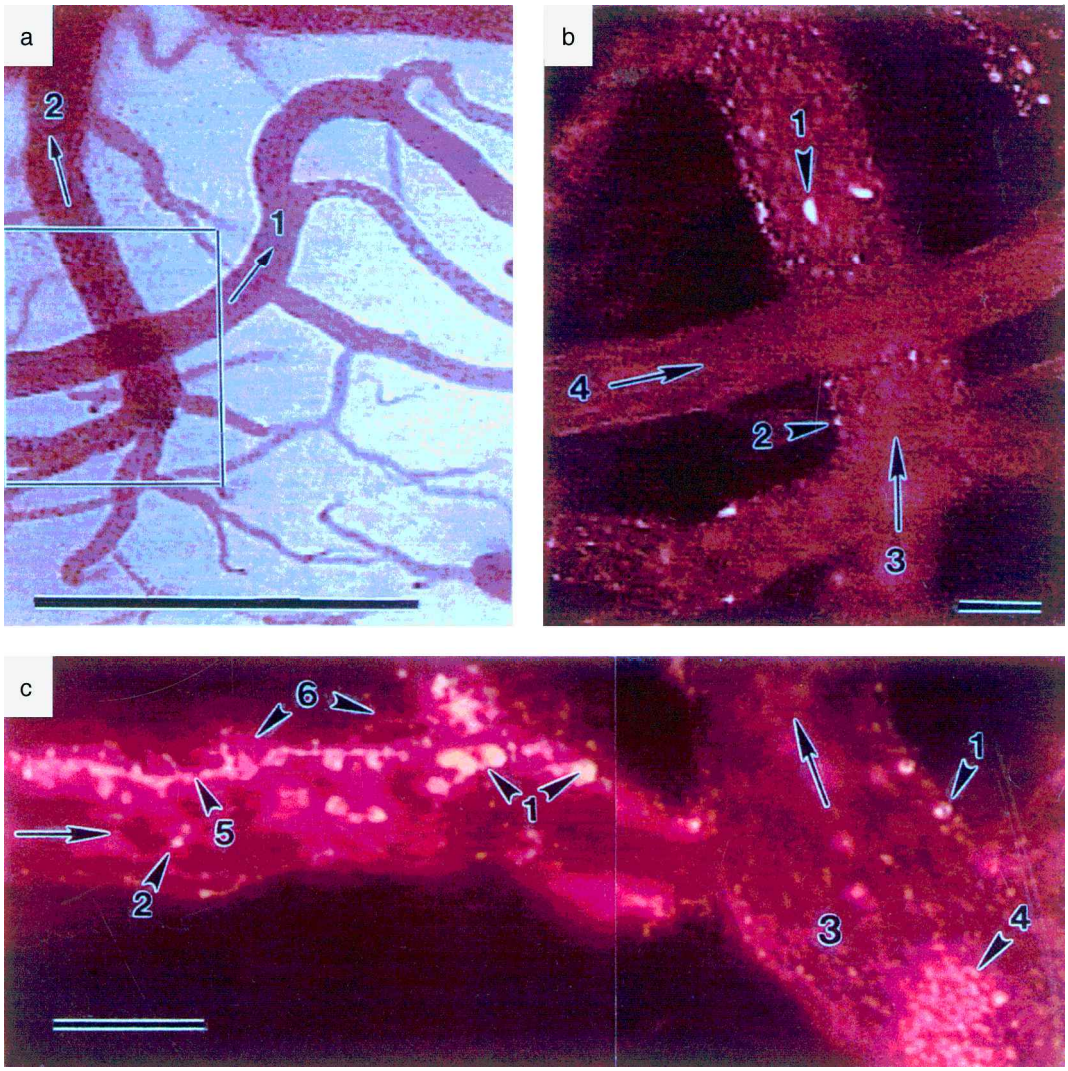

Figure 1 (a) Brain (leptomeningeal/pial) vasculature: video recording using epi-illumination under normal visible light. Arrows indicate the direction of blood flow. This is a normal representative pattern of leptomeningeal blood vessels observed with the cranial window technique. Arterioles (1), venules (2). Scale bar = 100 μm. (b) Brain: figure corresponds to the area marked by a rectangle in (a). The image was obtained under epifluorescent light. Results obtained with conjugated equine estrogens (CEE)- or progesterone-treated or untreated ovariectomized rats were identical. Images were completely normal with no detectable vascular reactivity. Rhodamine-tagged leukocytes (1) and platelets (2) are moving slowly with blood flow in the venule (3). They are not seen in the arteriole (4) because of the rapid rate of blood flow. Endothelial cells of the vascular walls are intact and are not rhodamine-tagged. Scale bar = 20 μm. (c) Brain: the leptomeningeal venules of medroxyprogesterone acetate (MPA)-treated ovariectomized rats were videotaped under epifluorescent light. Arrows indicate the direction of blood flow. Numerous rhodamine-tagged leukocytes (1) and platelets (2) are marginated and adhere to the venular wall. In the larger venule (3), there is an aggregation of platelets (4), forming a clot or thrombus. Endothelial lining is also rhodamine-tagged (5), and there is diffuse perivascular fluorescence (6), both indicating the breakdown of the blood–brain barrier. Scale bar = 10 μm

though the leukocytes and platelets were tagged with fluorescent rhodamine, they did not marginate, and there was no clot formation. There was a complete absence of fluorescence in the endothelial lining, and there was no diffuse fluorescence in the perivascular space. Similarly, estrogens (CEE and 17β-estradiol) did not produce any detectable vascular reaction. Pretreatment of ovariectomized rats daily for 3 weeks with 2 mg/kg of oral CEE did not prevent the vascular disruption produced by the acute administration of MPA. Intravenous administration of 10 μg of MPA produced a rapid vascular response, whereas, even at the 100-μg dose,

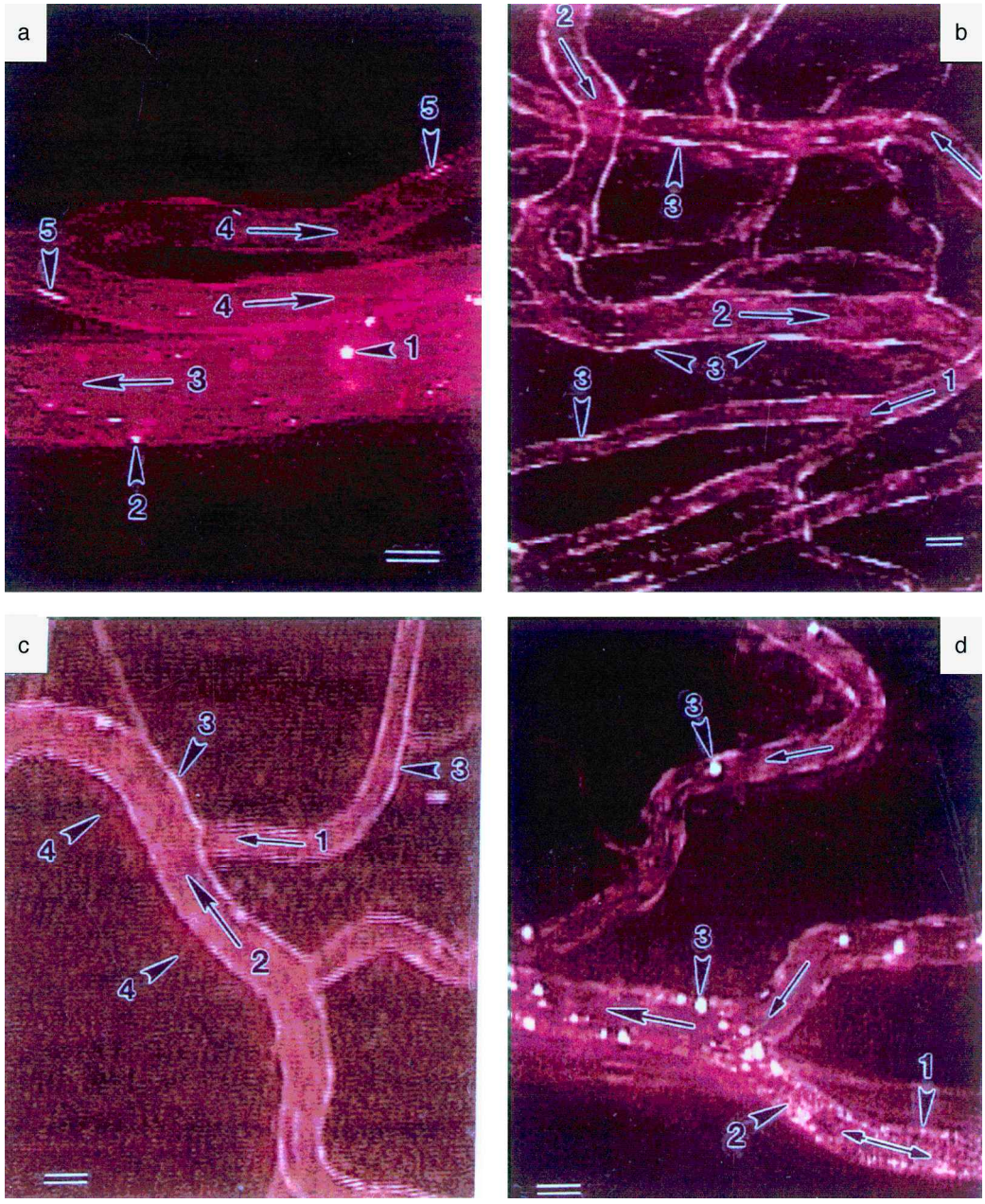

Figure 2 (a) Mesenteric vasculature: epifluorescent light images of ovariectomized control rats or rats treated with conjugated equine estrogens (CEE) or progesterone. Arrows indicate the direction of blood flow. This is a normal image without any vascular disruption. Rhodamine-tagged leukocytes (1) and platelets (2) are moving slowly with blood flow in the venule (3). In the arteriole (4), two leukocytes (5) are seen as short rod-shaped structures owing to the rapid rate of blood flow. Endothelial cells of the vascular walls are intact and do not show any rhodamine uptake. Scale bar = 10 μm. (b) Mesenteric vasculature: images were obtained under epifluorescent light in ovariectomized rats treated with medroxyprogesterone acetate (MPA). Arrows indicate the direction of blood flow in arterioles (1) and venules (2). Rhodamine-tagged endothelial cells (3) are seen in both arterioles and venules. This is an indication that MPA has damaged the endothelial lining of the blood vessels. Scale bar = 10 μm. (c) Mesenteric venules: images were obtained under epifluorescent light in ovariectomized rats treated with norethindrone. Arrows indicate the direction of blood flow in postcapillary venules (1) and large venules (2). Damage to blood vessels is similar to that seen in MPA-treated animals, with distinct rhodamine tagging of endothelial lining (3). There is also faint diffuse perivascular fluorescence (4). Scale bar = 10 μm. (d) Mesenteric venules: images were obtained under epifluorescent light in ovariectomized rats treated with norethindrone. Arrows indicate the direction of blood flow. One venule (1) is in a state of hemostasis (arrow points in two directions) owing to aggregation of platelets, forming a thrombus (2). Numerous rhodamine-tagged leukocytes (3) adhere to the endothelial lining, which also shows fluorescence. Scale bar = 10 μm

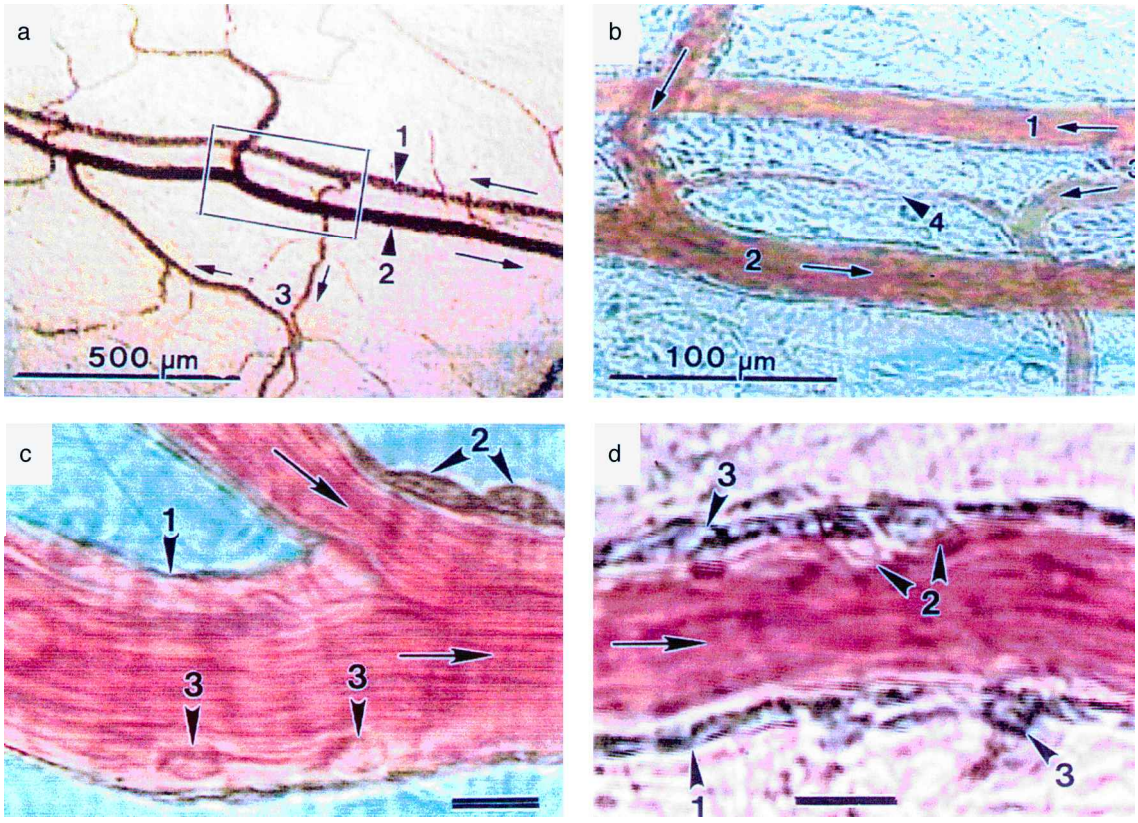

Figure 3 (a) Mesenteric vasculature: video recording of mesentery under light microscopy. Arrows indicate the direction of blood flow. Overview of typical untreated mesenteric microvascular bed with arteriole (1), venule (2), precapillary and postcapillary loops (3) shown. Scale bar = 500 µm. (b) Mesenteric vasculature: light microscopic recording of enlarged view of the rectangle in (a). Arrows indicate the direction of blood flow. Arteriole (1), venule (2), precapillary arteriole (3), arteriovenous capillary (4). Scale bar = 100 µm. (c) Mesenteric venule: light microscopic recordings of control or estrogen- or progesterone-treated ovariectomized rats. Figure shows completely normal venule. Endothelial cells (1) lining the blood vessels are not apparent, but two pericytes are visible (2). Several leukocytes (3) roll along the endothelial lining, a normal phenomenon in venules. Scale bar = 10 µm. (d) Mesenteric venule: light microscopic recording of norethindrone- or medroxyprogesterone acetate (MPA)-treated ovariectomized rats. Identical effects were obtained with both progestins. Figure shows severely damaged, inflammatory venule, judging by the thickened endothelial lining (1), adhering leukocytes (2) and several transmigrating leukocytes (3). Scale bar = 10 µm

progesterone did not induce a reaction. The toxic effects of MPA were also observed in non-ovariectomized animals.

DISCUSSION

This study was conducted in animals as we were exploring toxic vascular actions of progestins. Moreover, the imaging technique has been well characterized in animals[24–26]. The progestins produced rapid vascular reaction in both peripheral and cerebral vasculature of all animals tested. The margination, adherence and transmigration of leukocytes and platelets are early events in atherogenesis, inflammation and thrombosis[27–29]. The intense fluorescence of the endothelial lining is interpreted as a sign of damage to the cell membrane and organelles of the endothelial cells. The diffuse perivascular fluorescence indicates that the permeability barrier of the vascular wall has been breached. Such a reaction in the cerebral blood vessels indicates a breakdown of the blood–brain barrier. The rapid vascular response in less than 10 min is suggestive of a non-genomic action of progestins. Since progesterone does not produce a toxic vascular response, the actions of progestins do not seem to be mediated through the progesterone receptor. The predominant action of progestins in the venules indicates an abundance of receptor sites or progestin-responsive elements in venules. Natural progesterone or estrogens did not demonstrate any vascular toxicity. Moreover, pretreatment

with estrogen did not prevent the deleterious vascular actions of progestins. Based on these findings, we conclude that the most commonly used progestins in oral contraceptive and HRT preparations may induce deleterious vascular actions. Such actions of progestins may account for many of the adverse events observed in clinical trials such as the WHI[16] and WHIMS[17,18].

Progestins could initiate the adverse events associated with HRT through a number of vascular actions. Vascular endothelial cells lining the blood vessels provide a non-adhesive surface to circulating leukocytes and platelets, and also prevent clot formation. Endothelial cell injury is a stimulus for atherosclerosis, inflammation and thrombosis[25–29]. Progesterone has a normal function in menstruation, which includes enhanced blood vessel fragility and bleeding[30,31]. Systemic exposure to high doses of progestins may affect vascular integrity in tissues other than the endometrium. Our results show clearly that currently used progestins induce endothelial damage, leading to inflammation and thrombosis in the periphery and the brain. Inflammatory processes play a major role in the pathology of both vascular disease and Alzheimer's disease, and both conditions will be exacerbated by progestins.

Clotting abnormalities – venous thrombosis, pulmonary embolism and other thromboembolic events – were the primary events in the decision to terminate the WHI study. Major factors that contribute to the development of venous thrombosis are vessel damage, hypercoagulability, inflammation and hemostasis or low blood flow. Progestins in oral contraceptives and HRT significantly increase the risk of venous thromboembolism, hemostasis and atherosclerosis[31]. Platelets play a prominent role in the initiation and propagation of thrombosis. We show here that progestins promote platelet aggregation and clot formation, which may contribute to the increased incidence of thromboembolism, stroke and dementia associated with HRT.

A two-fold increase in ischemic stroke in the estrogen plus progestin group in the WHI study[18] could be initiated by the cerebrovascular actions of progestin, as seen in our study. Furthermore, the endothelial damage would disrupt cerebral blood flow, induce inflammatory damage and exacerbate amyloid-β toxicity[24], leading to increased risk for vascular dementia as observed in WHIMS. Progestin in the HRT formulation used in these studies might also oppose the beneficial actions of estrogens[2], and may accelerate pre-existing Alzheimer's disease pathology. With advanced age, the progressive brain damage in dementia, even though clinically not evident, may cause significant tissue damage so that either hormones[15,16] or non-steroidal anti-inflammatory agents[32] may not reverse the dysfunction. In postmenopausal women, the vascular actions of progestin may also accelerate the progressive course of the disease, contributing to the increased incidence of dementia observed in WHIMS.

The precise mechanism of the acute vascular response induced by progestins remains to be established. We have previously demonstrated that the vascular disruption in this model is mediated by oxygen radicals and proinflammatory cytokines[33]. Divergent effects of progesterone and MPA on cellular signalling pathways were shown by a recent study[34] . In hippocampal neuronal cell cultures derived from fetal rat brain, estradiol and progesterone were found to have a protective effect against glutamate neurotoxicity, whereas MPA had no protective effect. The neuroprotecive effect of steroid hormones involves the activation of extracellular signal-regulated kinase (ERK) and the transduction of the phospho-ERK signal to the nucleus. Even though estradiol, progesterone and MPA activated ERK, only estradiol and progesterone caused translocation of ERK activation to the nucleus. Furthermore, estradiol-induced nuclear translocation of ERK was blocked by coadministration of MPA.

We observed divergent vascular responses to estrogens, progesterone and progestins in a live animal. In our model, progestins initiated cytotoxic effects which were not prevented by estrogens. We speculate that the vascular actions of progestins may involve the activation of p38 mitogen-activated protein kinase (MAPK)[35] in endothelial cells, stimulating the expression of adhesion molecules, activation of leukocytes and up-regulation of proinflammatory cytokines.

The selection of MPA as the progestational agent in HRT may have been based mainly on endometrial actions of the compound, and not based on its systemic effects. Progestins such as MPA antagonize the beneficial actions of estrogens with regard to lipid changes, atheroma development[36] and vascular reactivity[37], whereas natural progesterone does not have this antagonistic effect. Prolonged exposure to progestins in HRT formulations may cause vascular dysfunction, leading to complications. Endothelial disruption caused by progestins may provide a nidus for atherosclerosis and thrombosis, and may be exacerbated by other risk factors, including mutations of coagulation factors. Even though

this study reports deleterious actions of progestins in an animal model, we propose that similar actions are feasible in menopausal women. It is possible that the vasculature in some postmenopausal women may be hypersensitive to the deleterious actions of progestins, in a manner similar to that in the animals in our study, owing to either genetic or metabolic vulnerability.

The publication of the WHI trial and the subsequent media publicity led to one of the most dramatic changes in medical treatments in modern history. It shook the foundations of postmenopausal health care and left women and their physicians searching for safer alternatives to hormone therapy. A number of natural and synthetic alternatives to hormones are being promoted without much evidence of clinical efficacy. Previous reports of adverse vascular actions of progestins were not given much credence owing to the accepted paradigm that progestins exert hormonal effects only on the reproductive organs. Our results suggest that the use of natural progesterone or progestins without serious vascular toxicity, combined with low doses of estrogens, may still be a viable option for some postmenopausal women, until safer alternatives are properly evaluated.

Conflict of interest T.T. and J.R. have received research support and honoraria for lectures from Wyeth Pharmaceuticals. L.C. and A.G., nil.

Source of funding This study was supported in part by the Department of Anatomy, USF College of Medicine and T.T.

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