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Tobacco smoke: A critical etiological factor for vascular impairment at the blood–brain barrier

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Abstract

Active and passive tobacco smoke are associated with the dysfunction of endothelial physiology and vascular impairment. Studies correlating the effects of smoking and the brain microvasculature at the blood–brain barrier (BBB) level have been largely limited to few selective compounds that are present in the tobacco smoke (TS) yet the pathophysiology of smoking has not been unveiled. For this purpose, we characterized the physiological response of isolated human brain microvascular endothelial cells (HBMEC) and monocytes to the exposure of whole soluble TS extract. With the use of a well established humanized flow-based *in vitro* blood–brain barrier model (DIV-BBB) we have also investigated the BBB physiological response to TS under both normal and impaired hemodynamic conditions simulating ischemia. Our results showed that TS selectively decreased endothelial viability only at very high concentrations while not significantly affecting that of astrocytes and monocytes. At lower concentrations, despite the absence of cytotoxicity, TS induced a strong vascular pro-inflammatory response. This included the upregulation of endothelial pro-inflammatory genes, a significant increase of the levels of pro-inflammatory cytokines, activated matrix metalloproteinase, and the differentiation of monocytes into macrophages. When flow-cessation/reperfusion was paired with TS exposure, the inflammatory response and the loss of BBB viability were significantly increased in comparison to sham-smoke condition. In conclusion, TS is a strong vascular inflammatory primer that can facilitate the loss of BBB function and viability in pathological settings involving a local transient loss of cerebral blood flow such as during ischemic insults.

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Disclosure of potential conflict of interest

Dr. Cucullo owns stock or stock options in Flocel Inc. as a founder and for activity related to the development of *in vitro* BBB models.

Keywords

Cerebral blood flow; Shear stress; Tobacco; Systemic; Public health; *In vitro*; Smoking; Ischemia; Atherosclerosis; Inflammation

1. Introduction

Tobacco smoke contains over 4000 different chemicals, many of which have adverse effects on human health and can contribute to the development of diseases such as stroke, lung cancer, and heart disease. Several studies have assessed that smoking makes a significant and independent contribution to the general risk of stroke and specifically to brain infarction. Smoking increases the risk of stroke by approximately 50% (Mannami et al., 2004; Shinton and Beevers, 1989) and the risk factor increases proportionally with the number of cigarettes smoked (Gill et al., 1989).

Smoking is also associated with a number of physiological changes (particularly in blood lipids and homeostatic factors) that help to explain its role in cerebrovascular disease. For example, increased blood viscosity may occur in smokers, leading to potential impairment of blood flow. This becomes particularly dangerous for the integrity of the brain microvasculature where vascular tone regulatory mechanisms are absent. It becomes even more crucial if the tight junctions (TJ) are already compromised by other concomitant pathological stimuli, whether or not they are exogenous or systemic/intravascular events. It is necessary that the BBB is strictly and precisely regulated in order to provide the homeostatic equilibrium required for the correct functioning of the brain.

Other studies have also shown a relationship between increased exposure to cigarette smoke and the presence of a silent cerebral infarction (SCI) that parallels the relationship between smoking and carotid atherosclerosis (Howard et al., 1998). The risk of SCI associated to smoking is substantial when compared to the effect of hypertension and other known cerebrovascular risk factors. The mechanism of increased stroke risk has been attributed to both pro-coagulant and atherogenic effects of smoking (Mast et al., 1998; Miller et al., 1998).

The numerous components of tobacco smoke may also favour atherogenesis by triggering a complex pro-inflammatory response that mediates leukocyte recruitment through upregulation of cytokine signaling and matrix metalloproteinase (e.g., MMP-2 and MMP-9) release (Nordskog et al., 2003; Nordskog et al., 2005). In fact, the level of the pro-inflammatory cytokines IL-6, tumor necrosis factor TNF- α , and IL-1 β significantly increased in 120-min side-stream cigarette smoke exposed mice (Zhang et al., 2002). IL-1 β , IL-6, and TNF- α play a major role in the inflammatory response of the vascular endothelium, which responds by upregulating the expression of selectins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Kaplanski et al., 1998). These adhesion molecules then promote the adherence of circulating immune cells to the luminal endothelial surface and propagate the vascular inflammatory response (McMullen et al., 2000). All together, this data points to the ability of cigarette smoke to modulate the complex interplay of signaling and adhesion molecules that control the vascular inflammatory response and therefore increasing the risk for the pathogenesis and progression of atherogenesis and vascular impairments.

While this data does not directly refer to the cerebrovascular system, it is logical to formulate the hypothesis that smoking can similarly affect the brain capillaries and the BBB.

Other works have revealed that nicotine, one of the main components of tobacco smoke, affects endothelial tight junctions (Abbruscato et al., 2002) by decreasing the expression ZO-1, occludin, cadherin, and adherent junctional proteins (Hutamekalin et al., 2008). Damage to the brain-to-blood $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter located on the abluminal surface of BBB has also been reported (Abbruscato et al., 2004). In addition, nicotine seems to facilitate focal ischemic brain (Wang et al., 1997), to affect the vascular tone, and to promote a variety of brain microvascular hemodynamic changes including decreased cerebral blood flow and hypertension (Argacha et al., 2008; Meyer et al., 2000).

Despite the clear association between smoking and vascular impairment (Rahman and Laher, 2007), the exact pathophysiology of TS exposure at the BBB level has not yet been revealed. In this respect, we present herein the first study aimed at characterizing the response of the human BBB endothelium to the exposure of whole mainstream tobacco smoke extract and the pathophysiology of ischemic BBB dysfunction with and without the priming exposure to tobacco smoke.

2. Results

2.1. Chronic exposure to a low physiological concentration of tobacco smoke reduces BBB endothelial cell viability while it does not affect astrocytes or monocytes

Compounds contained in TS may affect the viability of cells comprising the BBB and trigger an inflammatory response that, in turn, may further lead to loss of BBB integrity. In this context, we evaluated the direct effect of chronic TS exposure (1 h every other hour for half a day over a period of 3 weeks) on the main cellular components of the BBB (EC and astrocytes). In addition, we evaluated TS effects on THP-1 cells, which are a well established and characterized human monocytic cell line successfully used by other groups in many *in vitro* experimental settings (Chen et al., 2008; Cicha et al., 2008; Fan and Karino, 2008). The experiments were performed in multiwell plates (BD Falcon cat.# 353046) under static conditions. Our data shows that TS exposure causes a significant loss of endothelial cell viability at 0.016 (down to $43.33\% \pm \text{SEM } 2.76$ from control) and 0.032 (down to $6.67\% \pm \text{SEM } 3.68$) but not at 0.008 puffs/ml (Fig. 1A). In contrast, astrocyte viability was only slightly reduced at the highest TS concentration (down to $89.35\% \pm \text{SEM } 5.63$) (Fig. 1B). THP-1 cell viability was not altered by TS exposure at any concentration (Fig. 1C). Our data corroborates a recent study demonstrating that astrocytes are more resistant than cerebral EC to genotoxic and cytotoxic effects (Bresgen et al., 2006).

Note that other experiments described in this work were performed with the use of the lowest TS concentration tested above (0.008 p/ml), which didn't affect the viability of the BBB endothelium. This decision was made to provide experimental conditions unbiased by the loss of endothelial viability and, therefore, closer to a real life situation in smokers (see also Supplemental information).

2.2. Tobacco smoke induces pro-inflammatory activation of brain microvascular endothelial cells

24 h of chronic exposure to the equivalent of 0.008 p/ml of TS caused a significant increase in the number of HBMEC cells expressing the pro-inflammatory adhesion molecules P-selectin, VCAM-1 and E-selectin (75, 124.6, and 63.7%, respectively) as compared to endothelial cells exposed to sham-smoke (Fig. 2A). In addition, TS exposure increased the medium concentration of the pro-inflammatory cytokines IL-6 and TNF- α (from 3.27 to 78.59 pg/ml and 19.80 to 45.47 pg/ml respectively) but did not significantly affect that of IL-1 β (Fig. 2B). The concentration of matrix metalloproteinases-2 (MMP-2) (from 1199 to 3228 pg/ml) as well as the activity were significantly increased, but no such change was observed with MMP-9

(Figs. 2C and D). Note that MMP-9 activity was undetectable by zymography. These experiments were performed under static condition.

2.3. Tobacco smoke induces macrophagic differentiation and activation of THP-1 cells

During infection or inflammation, circulating blood monocytes migrate into extravascular tissues where they mature into tissue macrophages. Following 24 h of exposure to 0.008 puffs/ml of TS, under static conditions, THP-1 cells showed signs of differentiation into a macrophagic phenotype, which is demonstrated by the increased number of CD14⁺ THP-1 cells (from 42.8 to 78.7%) over the total CD45⁺ population (Fig. 3A). Upon this differentiation, their immunoresponsive properties were also markedly enhanced as demonstrated by a significant increase in the release of IL-1 β (from 0.47 to 65.15 pg/ml/h) and TNF- α (from 0.63 to 147.06 pg/ml) versus sham-smoke controls (Fig. 3B). By contrast, IL-6 release was not affected. We also observed a significant increase in the concentration of MMP-2 (from 264 to 534 pg/ml) and MMP-9 (from 2653 to 6190 pg/ml) measured over the 24 h of exposure (Fig. 3C). The increased release of MMP-2 and MMP-9 was also paralleled by a significant increase in MMP activity as shown in Fig. 3D.

2.4. Tobacco smoke exposure promotes endothelial-THP-1 adhesion and synergistically increases the release of pro-inflammatory mediators

In this experiment HBMEC cells under static culture conditions were exposed to 0.008 p/ml equivalent of tobacco smoke or sham-smoke for 24 h. After 24 h of exposure, the TS medium was removed from the endothelial cultures and replaced with TS-free medium. THP-1 cells, separately exposed to 0.008 p/ml equivalent of tobacco smoke or sham-smoke for 24 h were first isolated by centrifugation from their original cultures and then resuspended (2×10^6 cells/ml) in wells containing endothelial cells. This established 2 parallel sets of co-cultures with HBMEC and THP-1 both previously exposed to TS or sham-smoke. THP-1 cells were left to adhere for one h without agitation. Non-adherent THP-1 were then washed away by gentle shaking. Adhesion of THP-1 cells to the vascular endothelium monolayer was dramatically increased in TS pre-exposed cultures in comparison to sham-exposed controls (Fig. 4A). In sham-TS conditions, the interaction between THP-1 and endothelial cells lead to a significant increase in the release of IL-1 β and TNF- α but not IL-6 (white bars) in comparison to that of the corresponding separated cultures. This synergistic potentiation is significantly increased by TS exposure (black bars) which also induced a significant increase in the release of IL-6 (Fig. 4B). A similar pattern was also observed concerning the release of MMP-9 and (to a lesser extent) MMP-2 (Fig. 4C). Note that the serum assessment of MMP-2 and -9 in HBMEC cultures returned negative results since the values were below above the minimum level of sensitivity of the detection method.

2.5. Under normal hemodynamic conditions TS does not affect BBB integrity but induces the vascular release of pro-inflammatory mediators

We used a well-characterized flow-based *in vitro* BBB model (DIV-BBB) which has been previously shown to closely mimic the physiology and functional characteristics of the BBB *in situ* (Cucullo et al., 2006; Cucullo et al., 2008). We established three parallel groups of four fully established (TEER $\sim 1200 \Omega \text{ cm}^2$, see Fig. 5-A1) DIV-BBB each that were perfused with THP-1 cells and exposed to different experimental paradigms. TEER monitoring performed during the 48 h prior flow-cessation/reperfusion (Fc/Rp) did not reveal any significant deterioration of the BBB caused by TS exposure (Fig. 5-A2). However, despite the lack of BBB deterioration, samples analysis showed a statistically significant increase in the serum levels of IL-1 β and TNF- α (Fig. 5B) that was paralleled by a similar increase in the level (Fig. 5C) and activity of MMP-2 and MMP-9 (Fig. 5D).

2.6. The loss of BBB integrity is significantly increased by the parallel exposure to tobacco smoke

Flow-cessation followed by reperfusion causes a biphasic opening of the BBB both *in vivo* (Huang et al., 1999) and *in vitro* (Krizanac-Bengez et al., 2006). Our results show that, in comparison to sham-exposed condition, (Fig. 6A, blue line) tobacco smoke significantly increased the magnitude and the duration of BBB integrity degradation (Fig. 6A, red line). The quantification by graphical integration of the TEER patterns revealed a loss of BBB integrity of $43.9\% \pm \text{SEM } 2.5$ in TS-exposed modules versus $25.3 \pm \text{SEM } 1.9$ in sham modules (Fig. 6B). These values were calculated by integrating the area under the curve segments defined by TEER values $\leq 600 \Omega \text{ cm}^2$. This TEER value has been previously shown to correlate with the presence of a leaky barrier (Cucullo et al., 2008).

The serum concentration and the activity of MMP-2 and MMP-9 were also significantly increased by TS over sham-exposed modules (Figs. 6C and D). Specifically, MMP-2 levels increased from 2260 pg/ml in control to 5355 pg/ml in modules subjected to Fc/Rp, and then to 8196 pg/ml when Fc/Rp was coupled with TS exposure. We also observed a sharp increase of MMP-9 which raised more than 2 fold (from 271 pg/ml, to 574 pg/ml) as a consequence of Fc/Rp and reached even higher levels in presence of TS (up to 907 pg/ml). The activity of MMP-2 and MMP-9 followed the same pattern and were significantly increased by Fc/Rp (290% and 206% respectively) over control. The exposure to TS increased their activity even further (up to 643% and 727% respectively) over controls. Note that these values are ≈ 3 times the activity levels observed in sham-exposed Fc/Rp modules.

The synergistic effect of tobacco smoke on BBB failure following flow cessation/reperfusion is also clearly demonstrated by the serum profile of IL-6, IL-1 β , and TNF- α in TS and sham-exposed modules versus control (Figs. 7A to C). TS not only altered the pattern of release of these cytokines but also significantly increased their total serum level (Fig. 7D). Specifically, IL-6 went from 73.05 pg/ml in control to 404.29 in Fc/Rp modules and moved further up to 567 pg/ml when Fc/Rp was coupled with TS. Similarly, TNF- α went from 80.59 in control to 266.48 pg/ml in Fc/Rp modules and up to 450 pg/ml in TS-exposed modules. Note that IL-1 β level was not affected by Fc/Rp but was however, drastically increased by over 35-fold (from 4.6 to 166 pg/ml) when Fc/Rp was paralleled by TS exposure. Note that both IL-1 β and TNF- α play a major role in the maintenance of BBB integrity by disorganizing the tight junctions between contiguous endothelial cells (Minagar and Alexander, 2003; Wong et al., 2004).

2.7. Transcriptional upregulation of pro-inflammatory genes is observed in BBB endothelial cells exposed to tobacco smoke

Six fully established DIV-BBB models were used (3 TS-exposed and 3 sham-exposed) for this experiment. By contrast with sham-exposed modules, gene array analysis of endothelial cells isolated from DIV-BBB modules exposed to non cytotoxic concentration (0.008 puffs/ml, 1 h every other hour for half a day over a period of 3 weeks) of TS showed a significant transcriptional upregulation of genes involved in the inflammatory response. These included specific chemokines (CCL2, CXCL1, CCL5, etc), pro-inflammatory cytokines (IL-8 IL-1 β , etc.) transmembrane receptors (CD14), transcriptional regulators (RELB, STAT3 etc), mitochondrial enzymes (such as superoxide dismutase 2, SOD2), and others (see Additional file 1 for details). Other upregulated genes of significant interest were the signal transducer and activator of transcription 3 (STAT3), which is an essential regulator of the anti-inflammatory function of ECs in systemic immunity. Apolipoprotein E (APOE) and serum amyloid 1 (SAA1) were also significantly upregulated. By contrast, the transcription of tissue inhibitor of matrix metalloproteinases-3 (TIMP 3) was significantly downregulated (≈ 2 fold) as well as that of claudin 5 (CLDN5, ≈ 3.7 fold), which is a key component of the inter-

endothelial tight junctions and plays a major role in the appearance of barrier properties in brain capillary endothelial cells.

3. Discussion

The BBB plays a critical role in maintaining brain homeostasis and also provides a very effective shielding against potentially harmful substances circulating in the blood that might otherwise, enter the brain. The BBB also provides a dynamic shielding for the brain against the body's peripheral immune defences.

In the forefront, separating the blood from the brain parenchyma, the BBB is directly exposed not only to dynamic hemodynamic changes but also to the effect of potentially noxious xenobiotic substances contained in tobacco smoke.

In this study we have shown that the exposure of cells to a physiologically non-toxic concentration of tobacco smoke induces a strong vascular inflammatory response at the cerebrovascular level that directly and independently affects the cerebrovascular endothelium and the circulating immune cells. This inflammatory activity, when paired with hemodynamic perturbation such as decreased cerebral blood flow commonly observed in chronic smokers, leads to exacerbated BBB damage. This suggests that tobacco smoke may contribute to an increased risk for cerebrovascular disease and ischemic stroke.

Several studies suggest an associative correlation between smoking and the increased risk for the pathogenesis of neuroinflammatory diseases such as multiple sclerosis and Alzheimer's disease (Almeida et al., 2008; Hawkes, 2007; Sundstrom et al., 2008). Our study provides additional data to support this correlation by linking the pro-inflammatory activity of tobacco smoke with the BBB, both at the endothelial and intravascular/immune levels.

At the endothelial level, the pro-inflammatory activity of TS further extends to the expression of the vascular endothelial adhesion molecules VCAM-1, E-selectin, and P-selectin in agreement with previous independent studies by Shen et al. (1996) showing that cigarette smoke condensate (CSC) can induce the expression of several adhesion molecules in human umbilical vein endothelial cells. This was paralleled by the release of TNF- α and IL-6 and that of matrix-degrading MMP-2. Since adhesion molecules regulate trafficking of lymphocytes and leucocytes across the endothelium into adjacent tissue, these inflammatory effects caused by the exposure to tobacco smoke may favour perivascular inflammation, loss of BBB integrity and significantly increase the risk for atherogenesis and eventually ischemic insult. The cytokines released by brain EC regulate redistribution of tight junctional proteins and affect the actin filaments regulating the cytoskeleton's junctional interactions (Gloor et al., 2001). The induction of vascular adhesion molecule expression (Kaplanski et al., 1998) promotes monocyte adhesion to brain EC and concurrently activates monocytes. In addition, these same cytokines mediate leukocyte recruitment to the central nervous system (Sellebjerg and Sorensen, 2003) and activate pro-inflammatory properties both of endothelial cells and the immune system thus leading to secondary, inflammatory BBB damage. Metalloproteinase activation facilitates extravasation of circulating immune cells across the BBB into the brain parenchyma (Gidday et al., 2005; Romanic et al., 1998). This data is in agreement with a previous study reporting increased levels of matrix-degrading metalloproteinases and pro-inflammatory changes in vascular EC exposed to cigarette smoke (Nordskog et al., 2003). Interestingly, this complex pattern of signaling, involving pro-inflammatory molecules (cytokines and chemokines) that mediate the progression of arterial lesions has been observed in larger peripheral vessels both *in vivo* and *in vitro* with the use of human aortic endothelial cells (Nordskog et al., 2005; Shen et al., 1996). This is however, the first time that similar underlying mechanisms of TS toxicity have been reported at the BBB level.

The pro-inflammatory activity of tobacco smoke exposure is also evident in the vascular endothelium at the gene transcriptional level. For example: RelB, an NF-kappaB transcription factor, which participates in the control of gene expression of many modulators of the inflammatory and immune responses (including the adhesion molecules E-selectin and intercellular adhesion molecule-1, ICAM-1) is significantly upregulated. Similarly, vascular adhesion molecules, chemokines, cytokines and other related pro-inflammatory genes were also significantly upregulated. This includes STAT3, a transcriptional regulator factor that through the IL-6/STAT3 signaling pathway (Horn et al., 2000) has been shown to play a major role as a key regulator of the inflammatory function of ECs in systemic immunity. STAT3 is also an angiogenesis modulator (Chen et al., 2008; Chen and Han, 2008) and acts as a molecular hub to link extracellular signals to transcriptional control of proliferation, cell cycle progression, and immune evasion.

Other genes upregulated by tobacco smoke exposure such as APOE (≈ 2 fold) and SAA1 (≈ 3.5 fold), are more directly related to atherosclerotic diseases and ischemic damage. Specifically APOE is responsible for the production of apolipoprotein E, which is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. This polymorphic gene has been studied for its role in several biological processes related to immunoregulation and is associated with elevated cholesterol and risk of atherosclerosis and ischemic stroke (Abboud et al., 2008; Eo and Kim, 2008).

In addition, SAA1 was also significantly upregulated. The transcriptional product of this gene is serum amyloid A (Xu et al., 2006). This is a potent chemoattractant factor that modulates the migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes (Badolato et al., 1994). The accumulation of amyloid deposits in cerebral vessels has been correlated with the presence of a leaky BBB (Schroder and Linke, 1999).

This data is of relevant importance because it shows for the first time that smoking can affect the transcription of genes involved in the pathogenesis of atherosclerosis and modulate an inflammatory response directly at the BBB level. This not only increases the risk for the development of cerebrovascular diseases but can also facilitate the loss of BBB integrity during ischemic insults. These findings not only outline the potential BBB toxicity of TS but also provide additional cues that may help to elucidate the complex vascular effects of smoking.

In synergism with the upregulation of pro-inflammatory genes, the downregulation of the transcription of tissue inhibitor of metalloproteinases (TIMP)-3 facilitates the post ischemic loss of BBB integrity caused by the enzymatic activity of matrix metalloproteinases (Butler et al., 1999; Candelario-Jalil et al., 2009).

CLDN5 a gene responsible for the transcription of claudin 5 was also significantly downregulated (≈ 3.7 fold) by TS exposure. This is a key element of the inter-endothelial tight junctions that are responsible for the maintenance of BBB integrity and functions (Koto et al., 2007; Ohtsuki et al., 2007). This finding provides additional cues of how smoking can negatively affect the BBB and increases the risk for cerebrovascular impairments.

TS induced a strong immune response in THP-1 cells even in the absence of immune-activated BBB endothelial cells. This suggests that smoking alone can act as an independent vascular pro-inflammatory primer capable to affect both the immune cells (or at list the monocytic subpopulation) and the vascular endothelium. THP-1 cells exposed to TS released significant amount of TNF- α and IL-1 β . These specific inflammatory cytokines negatively affected the integrity of the BBB by disorganizing cell-cell junctions, decreasing the brain solute barrier and enhancing leukocyte endothelial adhesion to the endothelial wall therefore, facilitating leukocyte migration into the brain parenchyma (Minagar and Alexander, 2003).

The release of TNF- α and IL-1 β was paralleled by that of MMP-2 and MMP-9, which activity level was also increased. These enzymes play a crucial role in the maintenance of BBB integrity. Particularly MMP-9, which is a metalloproteinases specifically associated with BBB opening in numerous neuropathological conditions including acute brain injury and cerebral ischemia (Ding et al., 2006; Kelly et al., 2006; Rosenberg and Yang, 2007; Shigemori et al., 2006; Svedin et al., 2007).

Our results also suggest that the exposure to TS may facilitate the differentiation of blood monocytes into tissue macrophages. This is a novel TS-dependent pro-inflammatory mechanism where both the immune cell differentiation and their inflammatory response are directly modulated by smoking. However, whether this is a generalized effect that extends to other immune cell phenotypes or is limited to the monocytic subpopulation is unclear and additional studies will be necessary to answer this question.

The vascular effects of TS are of paramount importance especially under conditions where BBB function and viability can be compromised by other systemic/pathological events such as sudden hemodynamic changes provoked by other pathogenic stimuli or by tobacco smoke itself (German and Logiiko, 1995; Koskinen et al., 2000), including decreased cerebral blood flow.

In this context, flow-cessation/reperfusion in the presence of circulating white blood cells has been shown to cause a transient opening of the BBB. This has been shown both *in vivo* (Huang et al., 1999) and *in vitro* (Krizanac-Bengez et al., 2006). Given these premises, flow cessation/reperfusion performed in dynamic *in vitro* BBB system provides a reliable way to assess the effect of an ischemic insult on the cerebrovascular system.

In this respect, despite the clear pro-inflammatory activity, tobacco smoke exposure alone was not sufficient to cause BBB disruption but when TS was coupled with a transient loss of blood flow, the loss of BBB integrity almost doubled in comparison to sham-exposed modules.

BBB failure was paralleled by an increased serum level of IL-6, IL-1 β , and TNF- α . The pattern of release of these pro-inflammatory cytokines followed the longitudinal time course and magnitude of BBB failure. These results show a clear correlation between smoking and the risk for the progression of BBB damage following an ischemic insult. This is of relevant importance since the loss of BBB integrity provides a temporarily unrestricted passage to the brain to potentially harmful substances circulating in the blood that may affect the neuronal function (DiNapoli et al., 2008) and to peripherally activated immune cells. This greatly increases the risk for secondary brain damage and may facilitate the pathogenesis of a variety of CNS diseases such as epilepsy (Marchi et al., 2007), silent cerebral infarction (Howard et al., 1998), hemorrhagic and non-hemorrhagic stroke (Gill et al., 1989), and small vessel ischemic disease (see Additional file 2).

In this study we have shown that tobacco smoke triggers a complex pro-inflammatory response at the BBB level, involving not only the immune cells but also the vascular endothelium. This is of significant importance since current evidence supports the idea that endothelial cells present at the vascular endothelium as well as at specialized high endothelial venules, play not only a critical role in the homing and recruitment of immune cells but can also influence the outcome of the immune response. We have also shown that the direct vascular damage imposed on brain EC by exposure to tobacco smoke is easily detected by the brain microvasculature, especially when paired with other pathological stimuli. Taken together, the upregulation of the transcription of APOE and SAA1, the endothelial inflammation, the significant loss of BBB integrity following flow cessation/reperfusion, and the increased reactivity of circulating immune cells strongly suggest that smoking may significantly contribute to increase the risk

for the pathogenesis of cerebrovascular diseases and the progression of vascular damage at the BBB level.

It remains for future studies to ascertain the variety of mechanisms by which tobacco smoke can simultaneously affect the BBB and the relative contribution of the large number of possible harmful substances such as formaldehyde, hydrogen cyanide, benzopyrene and many others of which tobacco smoke is highly enriched with (Torikai et al., 2005).

4. Experimental procedures

4.1. TS preparation

Concentrated smoke solution was prepared from 2R4F research cigarettes, which are high nicotine, high tar, filtered cigarettes (University of Kentucky). The mainstream smoke from 10 cigarettes was drawn by vacuum pump through 10 ml of sterile phosphate buffered saline (PBS) (abbreviated TS1) and then through another 10 ml of PBS (TS2) to form the stock smoke solutions, using a Borgwaldt RM2 apparatus. The Federal Trade Commission (FTC) standard smoking protocol with a 35 ml draw, 2 second puff duration, 1 puff per 60 s, was used. This protocol resulted in approximately 8 puffs per cigarette. Note that TS1 and TS2 fractions were combined together in order to obtain the TS solution used for the experiments described herein. The TS was quantified as puffs/ml (p/ml), so that the concentration of trapped smoke per cigarette was approximately 0.8 p/ml. The stock TS solutions (8.0 p/ml) were then stored at -80°C and freshly thawed and diluted (to 0.008, 0.016, and 0.032 p/ml) just prior to use. Prior studies showed that biological activity was stable at -80°C for at least 6 months and stable at 4°C for at least one week.

4.2. Cell culture

Normal adult human brain microvascular endothelial cells (HBMEC, cat# 1000), and human adult astrocytes (HA, cat# 1800) were purchased from ScienCell Research Laboratories, San Diego, CA 92121. HBMEC were initially expanded in 75 cm² flasks pre-coated with fibronectin (3 $\mu\text{g}/\text{cm}^2$) with the appropriate endothelial growth medium consisting of MCDB 105 (Sigma, Cat# M6395), 10% human AB serum (SIGMA, Cat# S-7148), 15 mg/100 ml of endothelial cell growth supplement (ECGS, Cat.# 1052), 800 U/ml of heparin (Sigma, cat# H3393), 100 U/ml penicillin G sodium and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate. HBMEC were characterized prior to use by immunocytochemistry with sheep polyclonal antibodies that recognized the human Von Willebrand Factor Antigen VIII (vWF/Factor VIII, US biological, Swampscott, MA, cat# F0016-13A).

HA were grown in Poly-D-Lysine pre-coated flasks (3 $\mu\text{g}/\text{cm}^2$) with Dulbecco's modified essential media (DMEM-F12) supplemented with 2 mM glutamine, 5% fetal bovine serum (FBS), 100 U of sodium penicillin G per ml, and 100 μg of streptomycin sulfate per ml. After reaching confluence, cultures were agitated overnight at 37°C . Cytosine arabinoside and L-leucine methyl ester (Sigma-Aldrich, MO, USA) were added in order to obtain a highly purified astrocytic population (Meyer et al., 1991). Immunological characterization, performed with rabbit polyclonal antibodies that recognized the glial marker GFAP (Dako Corporation, Carpinteria, CA, USA) showed that about 95% of living cells (as visualized by DAPI) were astrocytes (data not shown). Both endothelial cells and astrocytes were maintained at 37°C in a humidified atmosphere with 5% CO_2 . Cell growth was monitored every day by inspection with phase contrast microscopy. Note that cell cultures were not passaged more than twice in order to minimize cell dedifferentiation.

The human monocytic leukemia cell line (THP-1, Cat# TIB-202) was purchased from American Type Culture Collection (ATCC, Manassas, VA). THP-1 cells were grown at 37°

C in 95% air–5%CO₂ in basal medium (ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001) plus 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum as specified by the vendor.

4.3. DIV-BBB protocol

Primary brain EC and astrocytes were cultured in a dynamic model of the blood–brain barrier where vascular endothelial cells cultured in polypropylene hollow fiber capillaries are exposed to physiological levels of shear stress. In this BBB model, the vascular endothelium acquire BBB phenotypic characteristics that closely mimic the BBB *in situ* (Cucullo et al., 2006; Cucullo et al., 2008; Santaguida et al., 2006). Briefly, the luminal surface of the hollow fibers was pre-coated with 3 µg/cm² fibronectin to allow EC adhesion of EC. The abluminal surface was similarly pre-coated with 3 µg/cm² of poly-D-lysine to promote astrocyte adhesion. Endothelial cells were first introduced into the luminal compartment and allowed to adhere for 3 h without pulsatile flow. To maximize EC attachment, flow of the medium was initiated in the abluminal compartment for 24 h following EC seeding and then redirected intraluminally. The level of shear stress was initially maintained at 1–2 dyn/cm² for 24 h and then raised to a constant value of 4 dyn/cm². Astrocytes were seeded on the abluminal surface of the fibers three days after EC were loaded. Typically, two weeks of co-culture of these cells are required to establish a fully functional BBB with a TEER greater than 1200 Ω cm² above the baseline (measured as TEER in a fully pre-coated cell-free module). Cell growth and viability was assessed by glucose consumption and lactate production measurements (data not shown) performed on luminal media samples collected every 2 days. The analysis was performed with the use of a dual channel immobilized oxidase enzyme analyzer (YSI 2700 SELECT) with turntable (YSI Inc., Yellow Springs, OH) as previously described (Cucullo et al., 2006; Cucullo et al., 2008; Santaguida et al., 2006).

4.4. Static culture setup

Endothelial cells were seeded in 24 well plates (100,000/well) pre-coated with fibronectin (3 µg/cm²) to enhance cell attachment and proliferation. Cells were left to adhere for 48 h and then grown to confluence. Cell growth was monitored by inverted light microscopy. Cells were then exposed to three different concentration of TS solution, equivalent to 0.008, 0.016 and 0.032 puffs/ml respectively. Sham-smoke cultures (not exposed to TS) were used as control. In parallel cultures, EC were also exposed to THP-1 monocytes to assess THP-1 adhesion on the vascular endothelial monolayer and the release of pro-inflammatory mediators.

4.5. TEER measurement

TEER measurement provides a rapid, simple evaluation of the integrity of the DIV-BBB (Cucullo et al., 2005; Cucullo et al., 2002; Santaguida et al., 2006). We used a newly developed TEER measurement device that utilizes electronic multiplexing to test multiple cartridges in rapid succession and delivers data to a computer (details at www.flocel.com). We have previously verified that this method documents a strong inverse relationship between TEER and permeability across the EC monolayer in the DIV-BBB modules (Cucullo et al., 2006; Santaguida et al., 2006).

4.6. Measurement of cytokines and MMPs

Medium samples collected from the luminal compartment were centrifuged at 5000 g for 5 min and stored at –20 °C until analysis. Levels of IL-6, IL-1β, and TNF-α in the medium were measured by ELISA (cat# IB39673 [IL-6]; # IB39654 (IL-1β); # IB39699 (TNF-α); IBL America, Minneapolis, MN) according to the manufacturer's protocols. Final calculation of cytokine levels (in pg/ml) took into consideration time and volume of the luminal compartment according to the formula: $\{(V_{\text{total}} \times C_c) - [(V_n \times C_n) + (V_{\text{total}} \times C_p)]\} / T_c - T_p$, where *V* represents

added volume of media (ml); C refers to the concentration of a cytokine/MMP level (pg/ml); T is time of sampling (in fraction of days: c and p indicate the current and previous samples, respectively; and n represents cytokine values in the fresh medium added after each sampling.

4.7. Flow cytometry (FACS) and data analysis

TS-treated and non-treated endothelial cells were rinsed with PBS. Cells were then gently mixed with chelation buffer (1 mM EDTA prepared in $\text{Ca}^{++}/\text{Mg}^{++}$ free PBS) using a pipette tip, washed in FACS buffer (PBS+0.5% BSA+0.02% NaN_3) and resuspended to approx. 1 million cells/100 μl . Cells were incubated with fluorescein di-isothiocyanate conjugated anti-P-selectin, phycoerythrin-conjugated anti-E-selectin, or alkaline phosphatase conjugated anti-VCAM-1 (respectively, cat.#555524; # 551144; #551146; BD Biosciences, San Jose, CA) for 20 min in the dark. Unbound antibody was removed by several washes with FACS buffer. Cells were then resuspended in 200 μl of FACS buffer/sample for analysis and the data was calculated with the use of Flow Jo software 6.1.1 (Tree Star) for Mac OS X. THP-1 cell suspensions were similarly tested for CD45/CD14(cat. #340040; BD Biosciences).

4.8. Matrix metalloproteinase activity

Aliquots of medium collected from the luminal compartment were centrifuged at 14,000 g for 15 min at 4 °C and the cell pellet was discarded. The total protein content of the medium was determined by Bradford assay (Bio-Rad Laboratories Protein Assay Kit, Hercules, CA). Gelatin zymography of electrophoresed medium samples was carried out on 7.5% polyacrylamide gels copolymerized with 2 g/l 90 Bloom Type A gelatin from porcine skin (Sigma). After electrophoresis, gels were washed in Triton X-100 (2.5 ml/l) and incubated for 24 h (37 °C) in enzyme buffer (50 mM Tris-HCl, pH 7.5; 5 mM CaCl_2 ; 100 mM NaCl; 1 mM ZnCl_2 ; 0.2 g/l Brij®-35; 2.5 ml Triton X-100; and 0.02 g/l NaN_3). Gels were stained with 0.5% Coomassie Blue R-250. Lysis bands were measured densitometrically using gel analysis software (Phoretix™ 1D, Nonlinear USA, Inc., Durham, NC). Optical densities of different gels were normalized based on known quantities of the specific standards (MMPs) loaded along with the experimental samples.

4.9. Extraction of RNA from endothelial cells

Endothelial cells were purged from the DIV-BBB modules (4 TS-exposed and 4 sham-exposed) by gentle enzymatic dissociation using trypsin and 2 mg/ml collagenase and collected by centrifugation. Supernatant media was removed and cells were lysed with TRIzol® Reagent (Life Technologies Corporation, Carlsbad, CA) by repetitive pipetting. Approximately 1 ml of the lysing reagent was used per $5\text{--}10 \times 10^6$ cells. *Phase separation*: Samples were incubated for 5 min at 15 to 30 °C to permit the complete dissociation of nucleoprotein complexes. Next, 0.2 ml of chloroform was added per 1 ml of TRIzol® and incubated at 15 to 30 °C for 2 to 3 min. Samples were next centrifuged at 12,000 $\times g$ for 15 min at 2 to 8 °C. *RNA precipitation*: RNA from the aqueous phase was precipitated by mixing with 0.5 ml of isopropyl alcohol per 1 ml of TRIzol®. Samples were incubated at 15 to 30 °C for 10 min and centrifuged at 12,000 $\times g$ for 10 min at 2 to 8 °C. The supernatant layer was then removed and the RNA pellet was washed once with a solution containing 1 ml of 75% ethanol per 1 ml of TRIzol® then centrifuged 7500 $\times g$ for 5 min at 2 to 8 °C. The RNA pellet was then dried briefly (air-dry or vacuum-dry for 5–10 min) and redissolved in RNase-free water by rinsing the solution a few times, and incubating for 10 min at 55 to 60 °C.

4.10. RNA purification and gene analysis

RNA samples were run through Qiagen RNeasy columns (Qiagen USA, Valencia, CA) to remove any genomic DNA contamination. The purity of RNA was determined by measuring the absorbance of the sample diluted in a solution of 10 mM Tris-Cl, pH 7.5 buffer at the

wavelengths of 260 nm and 280 nm and calculating a ratio of these absorbance values. Pure RNA has an A260/A280 ratio of >1.90. Samples were then processed by Illumina bead-array based gene expression platform at Genomics Core Facility using HumanRefSeq8 BeadChips. Each of these 8 microarrays on the Human-8 Chip contains 23,000 oligonucleotide probes (corresponding to over 23,000 well-characterized genes). Transcription changes were analyzed by the use of Ingenuity Pathway Analysis (IPA) software (Ingenuity™ Systems, Mountain View, CA) For each experimental condition, genes were considered expressed in the specific population and included in the analysis only if they were flagged as present in all 3 replicates by the gene chip operating software, and if average normalized signal intensity minus background value was above an arbitrary cut off of 25 (according to the manufacturer protocol).

4.11. Statistical analysis

For parametric variables (e.g., TEER levels, glucose consumption, lactate production, cytokines levels), differences between populations were analyzed by ANOVA. *p* values < 0.05 were considered statistically significant. Bonferroni analysis was used to account for comparisons of multiple parameters among groups. For non-parametric indices (e.g. densitometries for zymogram), we used the Kruskal–Wallis test followed by Mann–Whitney *U*-test. We used 6 cartridges per data point in the experiments related to the effect of flow cessation/reperfusion (with and without the priming of tobacco smoke) on BBB integrity. Endothelial cells isolated from a group of 3 independent DIV-BBB per experimental condition were used for the gene array analysis. Based upon previous experiments, the number of DIV modules used for each of the experiments described above provided sufficient power to demonstrate statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.brainres.2009.06.033.

Fig. 1.

Effect of TS exposure on human BBB endothelial cells, astrocytes, and monocytes. Panel A: Chronic exposure to TS significantly decreases cell viability of BBB endothelial cells at concentration of 0.016 and 0.032 p/ml but not at 0.008 p/ml. The effect on astrocytes was negligible and only at the highest TS concentration (Panel B) while THP-1 viability was completely unaffected (Panel C). The asterisk(s) “*” indicate a statistically significant difference ($p < 0.05$) versus control.

Fig. 2.

TS exposure causes pro-inflammatory activation of endothelial cells. 24 h of exposure to a low concentration of TS (0.008 p/ml) significantly increased the number of individual human brain microvascular endothelial cells expressing the pro-inflammatory adhesion molecules P-selectin, VCAM-1, and E-selectin (Panel A). Furthermore, TS exposure induced the release of IL-6 and TNF- α but not IL-1 β (Panel B). This was paralleled by the release of MMP-2 (Panel C). Note also, that only MMP-2 activity was detectable by zymography (Panel D). The asterisks indicate a statistically significant difference ($p < 0.05$) versus the corresponding control.

Fig. 3.

TS facilitates maturation of monocytes and their immune response. Chronic TS exposure of THP-1 cells induces their maturation into a macrophagic phenotype as demonstrated by the increased number of cells positive for CD14 (Panel A). In addition, TS exposure induced a marked increase of IL-1 β and TNF- α release but not IL-6 (Panel B). Both MMP release and activity were increased by TS (Panels C and D). A statistically significant difference ($p < 0.05$) versus control is indicated by the asterisk “*”.

Fig. 4.

Synergistic effect of TS-mediated inflammatory activation on HBMEC in presence of THP-1 cells. TS exposure facilitates adhesion of THP-1 cells to the vascular endothelium monolayer (Panel A). By contrast to IL-6, IL-1 β and TNF- α levels measured when endothelial cells and monocytes are co-cultured together are significantly higher than those measured in the corresponding separate cultures (Panel B, white bars). This synergistic potentiation is significantly increased by TS exposure (Panel B, black bars). Measurement of MMP-2 and MMP-9 showed a similar pattern (C). The asterisk “*” indicates a statistically significant difference ($p < 0.05$) versus control while the double asterisk “**” indicates a significant difference versus the corresponding TS-exposed isolated cultures.

Fig. 5.

Under normal hemodynamic conditions TS does not affect BBB integrity but induces the vascular release of pro-inflammatory mediators. The DIV-BBB modules under pulsatile flow reach high level of TEER ($\approx 1150 \Omega \text{ cm}^2$, Panel A1). The exposure to tobacco smoke and in presence of circulating THP-1 cells does not affect the integrity of the BBB (Panel A2). However, despite the absence of BBB deterioration, TS exposure significantly increased the plasma concentration of IL-1 β and TNF- α (Panel B). This was paralleled by the release and activation of MMP-2 and 9 (Panels C and D).

Fig. 6.

The loss of BBB integrity is significantly increased by the parallel exposure to tobacco smoke. Flow-cessation reperfusion induces a biphasic opening of the barrier. The magnitude and the duration of the BBB opening is greatly enhanced by TS exposure (Panel A and B) Serum level (Panel C) and activity (Panel D) of MMP-2 and MMP-9 were also significantly increased during reperfusion and reached the climax in the TS-exposed modules The asterisk “*” and the plus sign “+” indicate a statistically significant difference ($p < 0.05$) versus control and sham-exposed modules undergoing flow cessation/reperfusion.

Fig. 7. TS affects the pattern of release of IL-1 β , IL-6, and TNF- α . (Panel A to C): The pattern of intraluminal release of IL-1 β , IL-6, and TNF- α matches the time course of BBB failure. (Panel D): The total serum level IL-1 β , IL-6, and TNF- α was also significantly increased by TS versus sham-exposed modules. The asterisk “*” and the plus sign “+” indicate a statistically significant difference ($p < 0.05$) versus control and sham-exposed modules undergoing flow cessation/reperfusion respectively.