Neutrophil extravasation in rat mesenteric venules induced by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), with special attention to a barrier function of the vascular basal lamina for neutrophil migration

Osamu Hoshi and Tatsuo Ushiki

Division of Microscopic Anatomy and Bio-imaging, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Summary. The present study was performed to investigate morphologically the process of neutrophil extravasation induced by the synthetic bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) in venules of the rat mesentery by the combined use of intravital microscopy and transmission electron microscopy (TEM). Special attention was given to the interaction of the neutrophils with the endothelial cells and endothelial basal lamina. By intravital microscopy, the rolling and adhesion of leukocytes were observed within 3 min in preparations activated by fMLP. Neutrophils remained in the vascular wall for more than 30 min even after penetration of the endothelium. In this period, neutrophils migrating between endothelial cells and their basal lamina were often observed by TEM. After 40 min application of fMLP, some of the migrating neutrophils parted from the vessel wall into the surrounding connective tissues. There were neutrophils which passed through the small pore of the basal lamina with a cytoplasmic constriction. These findings indicate that the endothelial basal lamina acts as a physical barrier against neutrophil extravasation, thus resulting in the transient retainment of neutrophils beneath the endothelial cells of the venular wall.

Introduction

At sites of acute inflammation, circulating neutrophils emigrate from the blood circulation into extravascular tissues through the postcapillary venules. This event starts with the attachment and rolling of neutrophils on the vascular wall, followed by their firm adhesion to the inner surface of the endothelial cells, and ends in the migration of neutrophils across the endothelium (Ohashi *et al.*, 1996). Recent studies have especially focused on the molecular events responsible for the interaction of the neutrophil to the endothelium and revealed that various kinds of adhesion molecules—including integrin and selectin—are involved in the adhesion of neutrophils (see the review by Albelda *et al.*, 1994).

The migration pathway of the neutrophils across the endothelium has been also investigated morphologically, mostly by transmission electron microscopy (TEM). Many classical studies have stated that neutrophils migrate through a junction between neighboring endothelial cells (an intercellular route: Marchesi, 1961; Florey and Grant, 1961; Hurley and Xeros, 1961), while some researchers proposed that neutrophils penetrate the cytoplasm of the endothelium (a transcellular route: Miyoshi *et al.*, 1970; Faustmann and Dermietzel, 1985; Bamforth *et al.*, 1997; Feng *et al.*, 1998). However, we recently succeeded in three-dimensionally demonstrating the migrating neutrophils by scanning electron microscopy (SEM) and clearly showed that the neutrophils migrate to the endothelium through a transcellular route in response to the peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Hoshi and Ushiki, 1998).

In the course of our previous studies, we noticed that

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Address for correspondence: Dr. Osamu Hoshi, M.D. Division of Microscopic Anatomy and Bio-Imaging, Niigata University Graduate School of Medical and Dental Sciences, 1 Asahimachi-dori, Niigata 951-8510 Japan
Tel: +81-25-227-2062; Fax: +81-25-224-1767
E-mail: o-hoshi@med.niigata-u.ac.jp

neutrophils often remain between endothelial cells and endothelial basal lamina, where these cells are covered with a thin cytoplasm of overlying endothelial cells and form a dome-like structure bulging into the vascular lumen. This generated the idea that the basal lamina of the endothelial cells might act as a certain barrier for neutrophil migration after the penetration of neutrophils to endothelial cells. Thus, the present study was undertaken to reexamine morphologically the process of neutrophil extravasation into the extravascular space. Special attention was paid to the role of the endothelial basal lamina in the process of neutrophil extravasation in acute inflammation. In the present study, we first observed the migration process of neutrophils in response to fMLP *in vivo* by intravital microscopy and then investigated neutrophils in each process by TEM. This study presents the evidence that the vascular basal lamina acts as a physical barrier for neutrophil migration across the vascular wall, thus resulting in neutrophil retention in the vascular wall.

Materials and Methods

Intravital microscopy

Adult male Wistar rats, weighing 150–200 g, were used in the present study. They were given standard laboratory animal chow and water *ad libitum*. After the animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal 50 mg/kg body weight), a mid-line incision about 1.5 cm in length was made through the abdominal wall, and a loop of the intestine was drawn from the abdominal cavity and pinned onto a plastic plate. The preparation on the plate was attached to a mechanical stage which was driven on a specially designed intravital microscope, and the exposed tissue was suffused with sterile Tyrode's solution maintained at 37°C. The mesenteric microcirculation was observed carefully by light microscopy using a water-immersion objective lens and transillumination with an optical light source. During the observation, fMLP solution (2×10^{-5} mol/l, 50 μ l) was applied to the microvasculature of the observation site with a 50 μ l micropipette. The optical images were projected to a television camera and recorded onto videotape using an S-VHS videocassette recorder.

Transmission electron microscopy

Ten to 60 min after the perfusion of fMLP or saline, the rats were perfused through the left ventricle with physiological saline followed by fixation with 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4). Immediately after perfu-

Fig. 1. Video photomicrographs of a venule 5 min (a) and 40 min (b) after fMLP application. **a:** A round neutrophil (arrow) is observed attaching on the vascular wall against the blood flow. **b:** Adhering neutrophils (arrows) can be seen on the vascular wall. Arrowhead indicates a neutrophil attaching to the outside of the vessel wall.

sion fixation, the mesenteric vessels were carefully excised from the body, and immersed in the same fixative for 12 h. The specimens were washed in a 0.1 M cacodylate buffer (pH 7.4) for 10 min, and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 90 min. They were dehydrated with a graded series of ethanol, transferred into propylene oxide, and embedded in Epon 812. Ultrathin sections were made using an ultramicrotome, stained with 0.5% tannic acid for 3 min, rinsed in distilled water, and further stained with 2% uranyl acetate and lead citrate. They were examined in a TEM (H7000, Hitachi, Tokyo) at 100 kV.

Results

Intravital microscopy

Under the intravital microscope, circulating leukocytes were identified as transparent globular structures in blood

Fig. 2. Transmission electron micrographs of a small venule after fMLP application. **a:** A neutrophil (Ne) adhering to the surface of the endothelium is found in the venule. E: erythrocyte. En: endothelial cell. $\times 10,200$. **b:** Closer view of a part of **a**. Arrow indicates a short projection of adherent neutrophil (Ne) inserting into the endothelial cell (E). Note also focal contacts (arrowheads) between the neutrophil and endothelium. $\times 30,000$

Fig. 3. Transmission electron micrographs of a venule after fMLP application. **a:** Two neutrophils are found in the venule. The upper neutrophil (Ne1) in the vascular lumen lightly attach to the endothelium (En) by several short processes, while the lower neutrophil (Ne 2) is located outside the endothelial sheet. $\times 10,000$. **b:** Closer view of the lower neutrophil in **a**. The neutrophil is located between the endothelial sheet (En) and its basal lamina (arrowheads). $\times 22,600$

Fig. 4. Transmission electron micrographs of neutrophil (Ne) passing through the basal lamina of the endothelium. **a:** The neutrophil constricts its cytoplasm at the site for traversing the basal lamina, thus forming a shape like an hourglass. En: endothelium. $\times 12,000$. **b:** Closer view of a part of **a**. Arrowheads indicate the basal lamina of the endothelium. The neutrophil appears to pass through a small pore of the basal lamina by forming a cytoplasmic constriction. Note the intercellular junction of the endothelium (arrow). $\times 40,000$

Fig. 5. Schematic representation showing the process of neutrophil extravasation induced by fMLP in the postcapillary venule. **A:** Rolling. **B:** Adhesion. **C:** Passing through the endothelium. Most of neutrophils take a transcellular route by penetrating the cytoplasm of the endothelial cells (Hoshi and Ushiki, 1999). **D:** Retainment between the endothelium and the underlying basement membrane. **E:** Passing through the basement membrane. Neutrophils pass through the pore of the basement membrane. **F:** Extravasation from vessel wall.

stream in the normal conditions. The adhesion of the neutrophils on the vascular wall was only rarely found before fMLP administration. The rolling of neutrophils was first observed in venules 3 min after fMLP administration, and some of the neutrophils then adhered to the endothelium against the blood flow (Fig. 1). The rolling and adhesion of neutrophils increased in number as time advanced. The adhering neutrophils remained attached to the surface of the vascular wall for more than 10 min; they sometimes left the vascular wall to enter the blood stream, while others continue to attach to the endothelium. After 40 min application of fMLP, many neutrophils were found attached to the vessel wall (Fig. 2) and often remained for longer than 30 min in the vascular wall, while some neutrophils in this period had left the vessels to extravasate in the connective tissue of the mesentery.

Transmission electron microscopy

By TEM, numerous neutrophils with a lobulated nucleus and typical cytoplasmic granules adhered to the endothelium of some venules in specimens 10–30 min after fMLP application. The cell body of these neutrophils came in to contact with endothelial cells by forming several focal attachments to the surface (Fig. 2, 3). A fine process or pseudopodium of the neutrophils was also sometimes found inserting itself into the thin sheet of the endothelial cells (Fig. 2). The cell membrane at the tip of this process appeared thick, suggesting the specialization of the membrane. Neutrophils piercing the endothelial cells were also found in the venules; these neutrophils lifted the endothelial sheet by pushing their body into the space between endothelial cells and their basal lamina, thus forming a dome-like structure consisting of a migrating neutrophil covered by a thin sheet of the endothelial cell (Fig. 3). Such neutrophils were often observed within 40 min after fMLP application, while neutrophils transverse the basal lamina

also appeared in this period; since the neutrophils passed the basal lamina through a small pore, they took the shape of an hourglass by forming a cytoplasmic constriction at the piercing point of the basal lamina (Fig. 4).

Discussion

The present study has demonstrated the process of neutrophil extravasation in the rat mesenteric venules by the combined use of intravital microscopy and TEM. Figure 8 is a schematic drawing of our findings on the process of neutrophil extravasation from the blood circulation to the extravascular space.

Our TEM investigations have shown that adherent neutrophils have several focal points for attaching to the endothelial cells. These focal attachments were probably produced by adhesion molecules such as E-selectin and integrin and have importance in resistance against the drag force exerted on the neutrophils by flowing blood (Ley, 2002).

In the present study, we have also revealed the pseudopodium of the neutrophils inserting itself into the cytoplasmic sheet of the endothelium. As already shown in our previous studies, neutrophils in response to the fMLP take a transcellular route, in which they penetrated the cytoplasm of the endothelial cells. Thus, it is probable that neutrophils first insert their pseudopodium into the endothelial cells which will pierce the endothelial sheet, resulting in the transcellular migration of neutrophils. In this context, neutrophils may simply push the thin sheet of endothelial cells mechanically with their processes, which enables the partial fusion of the luminal and abluminal cell membrane of the endothelial cells. On the other hand, some researchers pointed out the possibility that neutrophils insert their thin process into caveolae and pinocytotic vesicles to penetrate the endothelial cells (Kvietys and Sandig, 2001).

Many TEM researchers—including us—have observed neutrophils remaining between the endothelial cell and the underlying basal lamina (Miyoshi *et al.*, 1970; Faustmann and Dermietzel, 1985; Feng *et al.*, 1998; Hoshi and Ushiki, 1998). The present study by the combined use of intravital microscopy and TEM has revealed that neutrophils remain in the vascular wall between the endothelial cells and the underlying basal lamina for a rather long time (probably for more than 30 min). A similar finding was reported by Oda *et al.* (1992), who stated in their intravital microscopic studies that the process of the neutrophil extravasation could be divided into five steps: 1) rolling and 2) adhering to the endothelium, 3) passing through the epithelial layer, 4) remaining in the vascular wall, and 5) passing through the basal lamina.

The most noteworthy finding in this study is that neutrophils lodged between the endothelial cells and the basal lamina passed through a small pore of the basal lamina by a constriction of their cell bodies. Shaw (1980) reported in chemotactic-fragment-induced lung inflammation that polymorphonuclear leukocytes including neutrophils migrated into the interstitium through a small opening in the basal lamina. A similar finding was also demonstrated in an *in vitro* model of leukocyte extravasation using cultured monolayers of bovine microvascular endothelial cells (Furie *et al.*, 1987); they showed that neutrophils after migration across the endothelium are retained between the endothelium and its basal lamina and then penetrate the basal lamina to move into the underlying connective tissue. These findings suggest to us that the basal lamina acts as a physical barrier against neutrophil extravasation, resulting in the transient retainment of the neutrophils beneath the endothelial cells of the venular wall.

Neutrophils are known to contain a large number of enzymes (e.g., elastase, cathepsin G) that have the ability to degrade connective tissue-associated proteins, glycoproteins and proteoglycans. However, Huber and Weiss (1989) considered in their *in vitro* studies of cultured human umbilical veins that the loss of basal lamina integrity associated with neutrophil diapedesis was not dependent on neutrophil elastase or cathepsin G and was resistant to inhibitors directed against neutrophil collagenase, gelatinase and heparanase. These findings *in vitro* may support the idea that neutrophils have less potential for disrupting the basal lamina. Recently, several investigators pointed out the importance of platelet-endothelial cell adhesion molecule-1 (PECAM-1) for neutrophil migration through the perivascular basal lamina as well as through the endothelium (Wakelin *et al.*, 1996; Dangerfield *et al.*, 2002). PECAM-1 deficient animals show the ability of neutrophil transendothelial cell migration but have defects in neutrophil migration through the vascular basal lamina (Duncan *et al.*, 1999; Thompson *et al.*, 2001). On the other hand, Walker *et al.* (1995) stated in their TEM studies of the rabbit lung that neutrophils migrated through preexisting holes in the basal lamina of alveolar capillaries during streptococcal pneumonia. It is also probable that, if present, a tiny hole in the basal lamina could become a target site for neutrophils penetrating the basal lamina.

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