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Neutrophil haptotaxis induced by the lectin KM$^+$*

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KM$^+$ is a D-mannose binding lectin from *Artocarpus integrifolia* that induces neutrophil migration *in vitro* and *in vivo.* This attractant activity was shown to be caused by haptotaxis rather than chemotaxis. The inhibition by D-mannose of the neutrophil attraction exerted by KM$^+$, both *in vitro* and *in vivo*, supports the idea that haptotaxis is triggered *in vivo* by the sugar binding sites interacting with glycoconjugates located on the neutrophil surface and in the extracellular matrix. In the present study an *in vivo* haptotaxis assay was performed by intradermally (i.d.) injecting ^{125}I-KM$^+$ (200 ng), which led to a selective staining of loose connective tissue and vascular endothelium. The radiolabelled area exhibited a maximum increase (five-fold) in neutrophil infiltration 3 h after injection, relative to i.d. 200 ng ^{125}I-BSA. We characterized the *ex vivo* binding of KM$^+$ to tissue elements by immunohistochemistry, using paraformaldehyde-fixed, paraffin-embedded, untreated rat skin. Bound KM$^+$ was detected with an affinity-purified rabbit IgG anti-KM$^+$ and visualized with an alkaline phosphatase based system. KM$^+$ binding to connective tissue and vascular endothelium was inhibited by preincubating KM$^+$ with 0.4 m M D-mannose and was potentiated by heparan sulfate (100 µg ml^{-1}). An *in vitro* assay carried out in a Boyden microchamber showed that heparan sulfate potentiated the attractant effect of 10 µg KM$^+$ by 34%. The present data suggest that KM$^+$ induces neutrophil migration *in vivo* by haptotaxis and that the haptotactic gradient could be provided by the interaction of the KM$^+$ carbohydrate recognition site(s) with mannose-containing glycoconjugate(s) in vascular endothelium and connective tissue. Heparan sulfate would act as an accessory molecule, enhancing the KM$^+$ tissue binding and potentiating the induced neutrophil haptotaxis.

***Keywords*: KM$^+$ lectin, neutrophil attraction, neutrophil haptotaxis, heparan sulfate**

Introduction

KM$^+$, a D-mannose-binding lectin from *Artocarpus integrifolia*, induces neutrophil attraction by a haptotactic mechanism. It is a tetramer formed by the non-covalent association of 13 kDa polypeptide chains and has at least two sugar binding sites. The latter are directly involved in KM$^+$'s ability to induce neutrophil migration, since its action is inhibited by D-mannose, *in vivo* and *in vitro*. Since one site recognizes carbohydrate(s) on the neutrophil surface [1] the other could interact with glycoconjugate(s) in the extracellular matrix. This second interaction would provide the substrate bound gradient required to direct the neutrophil movement. A haptotactic mechanism is also used by C5a [2], casein [3] and IL-8 [4] to induce neutrophil migration *in vitro*. The neutrophil attraction exerted *in vitro* by IL-8 is potentiated by heparan sulfate, supporting the hypothesis that heparan sulfate can be the substrate for *in vivo* IL-8 tissue binding to form the haptotactic gradient [5]. Since KM$^+$ mimics the IL-8 effects on neutrophils [1, 6], in the present study we determined if KM$^+$ induces neutrophil haptotaxis *in vivo* and if the haptotactic gradient can be due to the interaction with heparan sulfate or other glycosylated components of vascular and perivascular tissue.

Materials and methods

Biological reagents

KM$^+$, a lectin from *Artocarpus integrifolia* was purified as described by Santos-de-Oliveira *et al.* [1]. Radiodination of KM$^+$ and bovine serum albumin (BSA) with ^{125}I was performed with Iodo-Beads® (Pierce Chemical Company, UK) according to the manufacturer's procedure. Anti-KM$^+$ antiserum was produced in rabbits. Anti-KM$^+$ IgG was purified by Protein-A-Sepharose® chromatography (Pharmacia LKB Biotechnology, UK) followed by affinity chromatography in a KM$^+$-agarose column. Heparan

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sulfate purified from bovine pancreas was kindly donated by Dr Helena B. Nader (Escola Paulista de Medicina, Federal University of São Paulo, SP, Brazil).

Tissue fixation and processing

Rat skin was fixed in 0.1 M sodium phosphate buffer, pH 7.4, containing 4% (w/v) paraformaldehyde (Sigma, USA) and 0.05% (w/v) glutaraldehyde (Polysciences, USA) for 24 h at 4 °C. The tissue was either embedded in paraffin or frozen in dry-ice-isopentane and 5 or 12 µm thick sections were collected on gelatin-chromalum subbed slides.

In vivo haptotaxis assay

^{125}I-KM^{+} (0.2 µg) in 100 µl phosphate-buffered saline (PBS) was injected intradermally (i.d.) into the dorsum of Wistar rats. As a control, ^{125}I-BSA (0.2 µg in 100 µl PBS) was injected contralaterally. Three hours later, the regions close to the injection site (1.5 cm in diameter) were excised, fixed and cut. Sections were coated with LM-1 photoemulsion (Amersham International Ltd, UK). After 2 days, the emulsion was developed and then stained with Hematoxylin and Eosin.

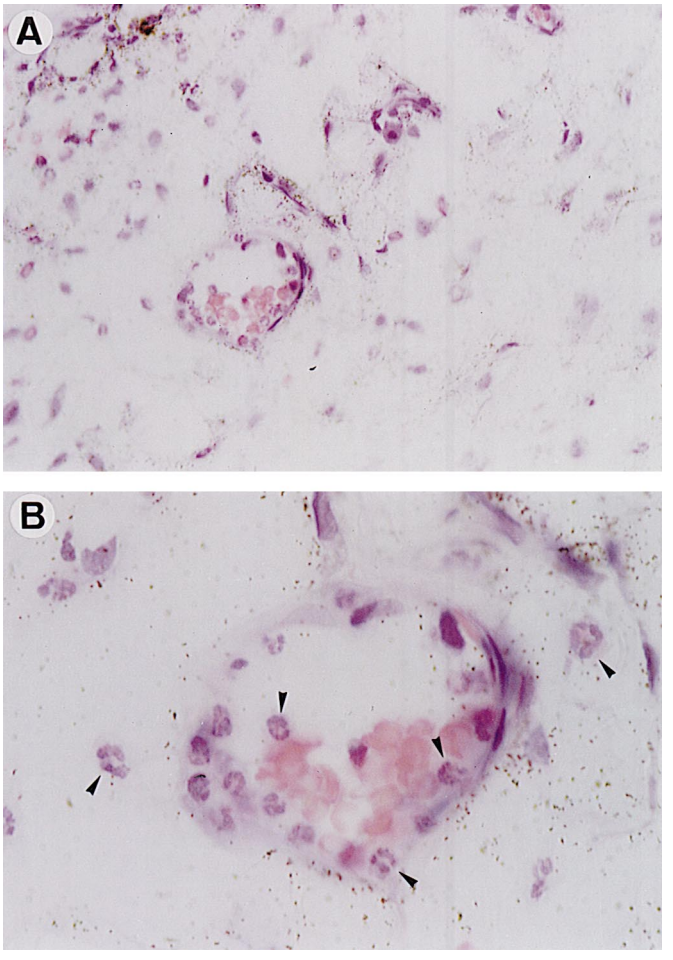

Figure 1. Autoradiographic evidence that ^{125}I-KM^{+} intradermally injected in rats binds to loose connective tissue in the perimuscular region (A, magnification 100 ×) and blood vessel wall (B, magnification 250 ×). The silver grains (dark dots) correspond to ^{125}I-KM^{+}; arrowheads, neutrophils.

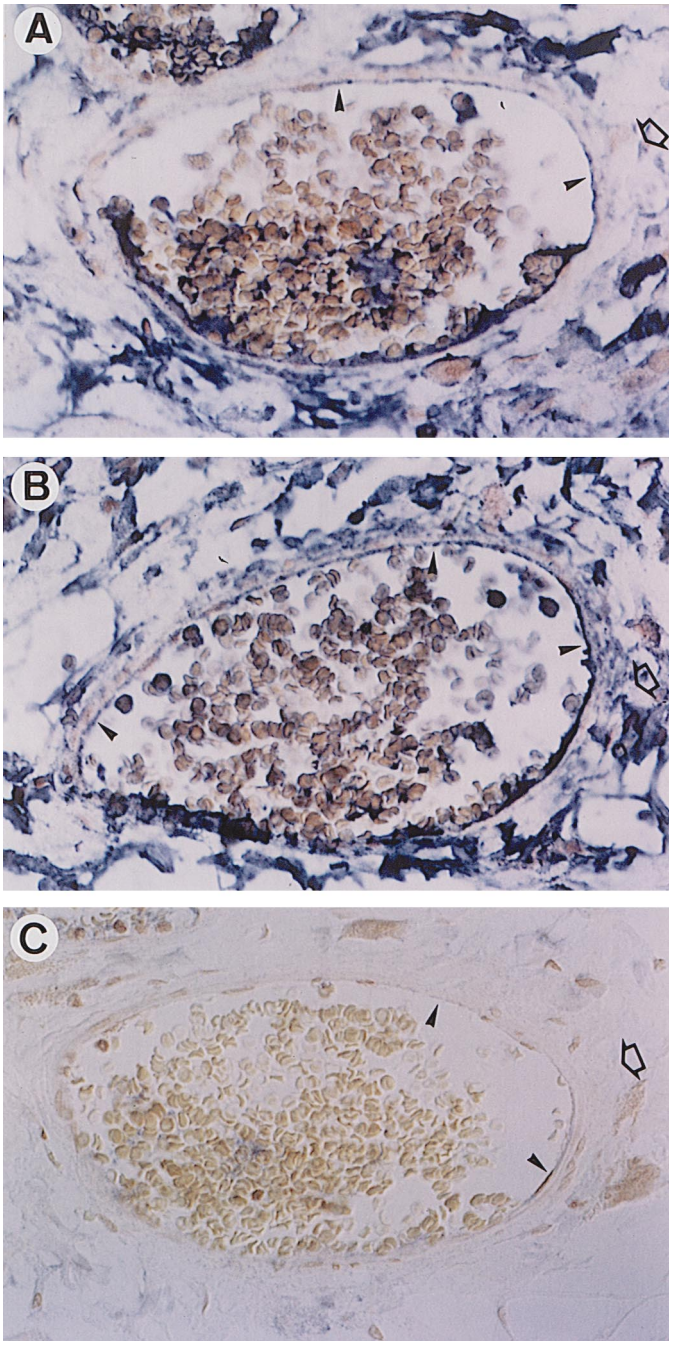

Figure 2. Immunohistochemical evidence that the KM^{+} binding to rat skin section (A) is potentiated by heparan sulfate (B) and inhibited by D-mannose (C). Sections were not stained in negative controls, which were obtained omitting KM^{+} from the incubation medium (not shown). Nomarski optics, magnification 125 ×. Open arrow, loose connective tissue; arrowheads, vascular endothelium.

In vitro neutrophil migration assay

A positive haptotactic gradient of heparan sulfate or chondroitin 4-sulfate (Sigma, USA) (0.8 to 100 μg ml^{-1}) was set up on a polycarbonate filter using a 48-well Boyden microchamber. After washing and drying, the filter was transferred to another microchamber with the more concentrated side facing the lower wells. BSA 0.01% (w/v) in RPMI medium was used to fill some lower wells to provide the controls, and 10 μg KM^+ in RPMI containing 0.01% (w/v) BSA was added to the remaining wells. Fifty microliters of the neutrophil suspension (10^6 cells per ml) were added to each upper well, and incubated for 45 min at 37 °C in a humidified atmosphere of air with 5% CO_2. The filter was removed, fixed and stained. The number of neutrophils that migrated through the filter was counted in five fields for each sample. Each sample was assayed in triplicate.

Ex vivo immunohistochemical KM^+ binding assay

KM^+ was diluted to 1 μg ml^{-1} in 20 mM sodium phosphate buffer, pH 7.4, containing 0.45 M sodium chloride and 5% (w/v) BSA (diluting buffer) with or without one of the following substances: heparan sulfate (100 μg ml^{-1}), chondroitin 4-sulfate (100 μg ml^{-1}), 0.4 M D-mannose, or 0.4 M D-galactose. Tissue sections (5 μm) were incubated with KM^+ with or without (control) each of the above substances for 1 h at room temperature. KM^+ was detected using an affinity-purified rabbit IgG anti-KM^+, diluted 1:50 in diluting buffer. IgG was detected using an anti-rabbit IgG, alkaline phosphatase-conjugated antibody (Promega, USA). Alkaline phosphatase was detected using nitroblue tetrazolium (Promega) and 5-bromo-4-chloro-3-indolylphosphate (Promega) [7]. The reaction was stopped with distilled water.

Results

Neutrophil haptotaxis induced *in vivo* by KM^+

The possibility that KM^+ could induce neutrophil haptotaxis *in vivo* was investigated by injecting ^{125}I-KM^+ intradermally into the dorsum of Wistar rats. Light microscope examination of the autoradiography of skin sections developed 3 h after injection showed that ^{125}I-KM^+ was concentrated in the loose connective tissue of the perimuscular region and vessel surface (Figure 1, panels A and B). ^{125}I-KM^+ was virtually absent in the dermis (data not shown). ^{125}I-BSA, used as control, was uniformly distributed throughout the section (data not shown). Neutrophil infiltrates were associated with the areas more intensely labelled with ^{125}I-KM^+. Photomicrograph B shows neutrophils apposed to the vascular endothelium and in the loose connective tissue. The largest number of infiltrated neutrophils occurred 3 h after injection of the attractant, when the number of neutrophils present in sections of skin treated with KM^+ was 82 neutrophils per mm^2 of loose connective tissue. This was five-fold higher than that observed in the ^{125}I-BSA control (15 neutrophils per mm^2).

Table 1. Heparan sulfate potentiates the *in vitro* neutrophil migration induced by KM^+

Treatment	*GAG concentration (μg ml^{-1})*	*Neutrophils/field*
KM^+	0.0	38.3 ± 2.7
KM^+ + heparan sulfate	0.8	39.0 ± 2.0
	4.0	32.6 ± 1.5
	20.0	51.4 ± 2.1*
	100.0	34.8 ± 1.0
KM^+ + chondroitin 4-sulfate	0.8	36.2 ± 2.0
	4.0	37.5 ± 2.1
	20.0	42.6 ± 2.0
	100.0	34.2 ± 1.2

Neutrophil migration in response to KM^+ (10 μg per well) in the absence or in the presence of heparan sulfate or chondroitin 4-sulfate. The number of cells counted per field for medium, heparan sulfate or chondroitin 4-sulfate in the absence of KM^+ were 14 ± 5, 15 ± 3, and 15 ± 6, respectively. Data are reported as mean number (± SEM) of neutrophils per field (15 fields).
*$p < 0.01$ compared to control (Student's *t*-test).

Effect of heparan sulfate on neutrophil migration induced *in vitro* by KM^+

To determine the possibility of the participation of heparan sulfate in haptotaxis, an *in vitro* migration assay was carried out in response to KM^+, in which the polycarbonate filter was first incubated with glycosaminoglycans. Heparan sulfate (20 μg ml^{-1}) potentiated 34% the neutrophil migration induced by KM^+, whereas chondroitin 4-sulfate (0.8–100 μg ml^{-1}) had no effect (Table 1). Neither heparan sulfate nor chondroitin 4-sulfate exhibited effect on neutrophil migration in the absence of the lectin.

Effect of heparan sulfate on the *ex vivo* binding of KM^+ to tissue structures

The hypothesis that heparan sulfate corresponds to a tissue ligand of KM^+ was tested in an immunohistochemical assay using KM^+ preincubated or not with heparan sulfate. Figure 2A shows the labelling observed when a skin section was incubated with KM^+ in the absence of glycosaminoglycan. Loose connective tissue and vascular endothelium were intensely immunostained. Heparan sulfate potentiated lectin binding to rat skin sections (Fig. 2B), as indicated by more intense labeling of tissue structures. Incubation of KM^+ with chondroitin 4-sulfate did not interfere with KM^+ binding to skin (data not shown).

Effect of D-mannose on the interaction of KM^+ with tissue structures

The involvement of the mannose recognition site in the binding of KM^+ to tissue components was assessed in a immunohistochemical assay using KM^+ preincubated or not with monosaccharides. D-mannose (0.4 M) abolished KM^+ binding to the entire extension of the skin section (Fig. 2C), whereas D-galactose (0.4 M) did not affect KM^+ binding (data not shown).

Discussion

We show here that the lectin KM^+ induces neutrophil migration *in vivo* by haptotaxis, since radiolabelled KM^+ intradermally injected in rats selectively bound to vascular and loose connective tissues. KM^+ binding was associated with neutrophil infiltration (Figure 1, panels A and B). Using a similar experimental design Rot [8, 9] showed that IL-8 binds to skin venules and veins. These tissue binding sites for both attractants may be involved in the promotion of neutrophil emigration by haptotaxis. Little is known about the molecular interactions occurring during neutrophil haptotaxis in the inflammatory process. The possible interactions that may permit the occurrence of neutrophil haptotaxis by IL-8 have been studied *in vitro*. Heparan sulfate potentiates the *in vitro* chemotactic activity of IL-8 towards neutrophils by three- to five-fold, whereas other glycosaminoglycans have no effect [5], supporting the idea that IL-8 interacts *in vivo* with tissue heparan sulfate, thus permitting neutrophil haptotaxis.

This observation stimulated us to determine if heparan sulfate also could potentiate the KM^+-induced neutrophil migration *in vitro*. A positive response, although smaller (34% potentiation), led us to use *ex vivo* assays, designed to identify if heparan sulfate could actually correspond to a tissue ligand of KM^+. The incubation of KM^+ with soluble heparan sulfate did not inhibit, but enhanced (Figure 2B), the lectin binding to vascular and connective tissue structures (Figure 2A). In contrast, incubation with soluble D-mannose completely abolished (Figure 2C) the KM^+ binding, suggesting that the tissue KM^+ ligand is a mannose containing glycoconjugate, different from heparan sulfate. The role of heparan sulfate interaction with KM^+ may be to enhance the interaction of the mannose-recognition site of KM^+ with mannose-containing tissue components during neutrophil haptotaxis. An analogous accessory role has been attributed to the heparan sulfate molecule concerning its interaction with basic fibroblast growth factor (bFGF), which has been demonstrated to be necessary for the binding of bFGF to its high-affinity receptor [10, 11].

The present observations lead us to suggest that: (1) the mannose recognition site of KM^+ is essential for tissue binding, and thereafter for neutrophil haptotaxis; and (2) the interaction of KM^+ with heparan sulfate, which is present on the vascular endothelium and underlying interstitium, may potentiate the neutrophil haptotaxis *in vivo*.

Acknowledgments

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