Transbarrier targeting in the intestine: nanomedical options in oncology

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Blood-tissue barriers

Each organ possesses its own particular barrier(s). We recently reviewed the blood-breast barrier, a complex and tightly regulated barrier, as well as one of the extremely “high” barriers, the blood-brain barrier [1] (the other two high barriers are in the testis and the placenta). Tissues and organs require organ-characteristic internal fluid environments to enable and facilitate their specific functions. For example, each glandular secretion is different in composition from all the others, and all are different from the blood; for the purposes of this review, the blood can be considered a homogenous fluid [1, 2]. To sustain these different milieux, structural and physiological barriers located at blood-tissue interfaces regulate bidirectional material flow between the tissue aqueous compartment and the blood compartment. The blood side of the barrier is demarcated by specialized structures and functions regulating the transfer of materials and cells; the luminal side of the barrier is demarcated by another set of structures and functions at the apical side of the epithelial cells, which regulate access of the luminal contents to the blood. Two barrier systems thus work in a coordinated fashion to elaborate a single consolidated barrier. 1. The blood-tissue barriers are of considerable biological importance, defining the operating environments of the organs. They are dynamic, biologically active interfaces, at which bidirectional exchanges of materials and cells are actively and continuously regulated (Figure 1). At its barrier, a tissue can accumulate particular molecules by taking these up from the blood, yet simultaneously it prevents the entry of other molecules which are also present in the blood. The separation between aqueous compartments results from the presence of tight intercellular junctions (occluding junctions) between cells, either between the endothelial cells in the microvessel wall, or between the characteristic epithelial cells of the organ. Tight junctions are an essential component of all the blood-tissue barriers (except in the placenta, where the barrier bases on a syncytiotrophoblast). The > 20 different protein constituents of the tight junctions, for example the claudins [3, 4], occludin [5], the cytoplasmic plaque proteins ZO-1,2,3, cingulin, and others [6] vary widely amongst tight junctions in different tissues. This, together with active regulation of tight junction assembly by zonula occludens [7], explains the variable paracellular permeability found in diverse tissues [6, 8, 9]. The separated aqueous compartments are bridged by selective uptake and transfer of specific molecules, mediated by a wide range of specific transporters and ion channels. Furthermore, specific enzymes may also be present to degrade any molecules (such as peptides) capable of crossing the barrier in spite of the tight junctions (Figure 1). The Figure shows both sides of the intestinal barrier, the blood side described above, and the luminal side which will be mentioned next. 2. The epithelial cells maintain uptake and transport systems at their apical side, quite different from those at the basolateral side. If a brush border is present, as in most gut segments, this will contain enzymes regulating passage of materials, and in the gut these may be digestive enzymes. The epithelium may coat itself with a continuous layer of viscoelastic gel in the form of a chemically sophisticated mixture of interacting molecules, of which the mucins are the major part, and in this case the barrier regulates passage across a mucous epithelium. Both the blood side and the luminal side of mucosal barriers will next be described in more detail for the intestine.

Blood-intestine barriers

Blood-intestine barriers regulate bidirectional passage of materials and cells between the blood and the intestinal lumen. These barriers have been particularly well-studied, because they are involved in the uptake of orally administered drugs. Studies have focused on
humans, rats and dogs in vivo, and researchers have developed a wide range of models in culture, including cell models, and several types of organotypic models [10]. The particular features of these barriers are therefore documented in a voluminous literature, and certain aspects of them are well understood. Blood-intestine barriers are particularly complex, being multilayered. Recent authors have indicated important roles for intestinal barriers in controlling the equilibrium between tolerance and immunity to environmental antigens, by regulating trafficking of antigens (macromolecules) through both transcellular and paracellular pathways to interact with the gut-associated lymphatic tissues; failure of the delicate balances between tolerance and immunity may underlie numerous diseases [9, 11, 12]. Figure 1 shows the several layers, from the capillary endothelial cells and the interstitium with its large populations of lymphocytes and fibroblasts, to the epithelial layer of enterocytes, and finally the double layer of mucus overlaying the enterocytes. The mucus layers are physicochemically complex, comprising mucin glycoproteins, trefoil peptides, and surfactant phospholipids [13, 14] and give the surface hydrophobic properties [15], preventing influx of water-soluble bacterial products and toxins [16]. The mucus is a viscoelastic gel, approximately 95% hydrated and expected to have diffusive properties generally in line with the Stokes-Einstein equation [17, 18, 19]; as will be noted below, however, nanoparticles much larger than expected can pass through mucus rapidly, indicating that mucus has unexpected properties. Barrier functions, both mechanical and physiological in the form of enzymes and pumps, are associated with each of the cell layers. Figure 1 indicates (incompletely) that, like all other mucosal barriers, the intestinal barriers interact closely with immune cells, and the mucus layers at the apical side contain antibodies, mainly IgA. In fact, the blood-intestine barriers regulate transfers of cells from the largest collection of immune tissues in the body [20]. Figure 1 also indicates (again incompletely) the complex regulation of the several functions of intestinal barriers. The regulation consists of multiple interlinked feedback control systems, including neural, hormonal and cytokine effectors with a wide range of less well characterized detectors. Neural regulation [20, 21] includes indirect control such as regulation of intestinal motility, but also direct control by axons secreting neurotransmitters directly onto the basal lamina of the epithelium [22, 23, 24, 25], thus influencing the epithelial cells and regulating their tight junctional apparatus [26]. Neural regulation may be complexed with immune regulation, via innervation of the immune cells present in the gut mucosa [27, 28, 29, 30]. Mast cells play a significant role in mediating the regulation by neurotransmitters and hormones [31, 32, 33, 34, 35, 36]. Hormonal regulation plays an essential role
in normal barrier function [32], and hormonal dysfunction underlies the effects of stress on gut permeability [37, 38]. Numerous cytokines also regulate the barrier, for example TNF-α, IFN-γ, IL-1β, IL-4, IL-13, prostaglandins, NGF, amongst others (well reviewed by Keita and Söderholm [20]), and act at both endothelial and epithelial levels [39].

Material transfers in both directions across the barrier characterize the gut barriers. This is evident especially in relation to the production of digestive enzymes, protons and bicarbonate ions in the enterocytes for secretion into the lumen. Simultaneously with the secretion of enzymes (passage of materials from blood to gut lumen) there may occur uptake of digested materials (passage of materials from gut lumen to the blood). Although common features characterize the family of intestinal barriers, each segment of the intestine has particular functions, which are reflected in the distinctive features of the barriers associated with the various gut segments. Each functional segment of the gut possesses its own characteristic barrier, adapted to the particular function of that gut segment, as discussed briefly here. The blood-intestinal barrier in the stomach was recognized first. As early as 1856 it was understood [40, 41] that the harsh digestive milieu of the stomach lumen necessitates protection for the stomach wall. The characteristic enzyme produced by the stomach enterocytes is pepsin in the form of pepsinogen, its production and function requiring separation of the stomach lumen from the blood compartment. More recently the protection of the enterocytes against pepsin and against back-diffusion of protons has been shown to reside in the (up to 450 µm) thick water-insoluble mucus layer [42, 43, 44, 45], which is impermeable to pepsin [44] and provides a space in which bicarbonate reacts with protons to ensure neutral pH at the surfaces of the enterocytes during acid secretion. This is aided by the secretion of neutralizing bicarbonate ions from the enterocytes [46, 47, 48], supported by the underlying capillary endothelial cells [49, 50]. The blood-intestinal barrier in the duodenum shares some features with that in the stomach, in particular the mucus layer, which is termed the “gastroduodenal mucus” layer [46, 47, 48]. However, the duodenum, unlike the stomach, takes up large amounts of materials from the aqueous environment of the lumen into the bloodstream, giving this barrier distinctive features. The duodenum is the major site for uptake of minerals, the best-studied being calcium [51] and iron [52]. Mechanisms for the uptake of iron into the duodenal enterocytes were controversial for many years and it is likely that some remain to be discovered; the presently identified separate uptake pathways are the Integrin-Mobilferrin pathway and the Divalent Metal Transporter-1 pathway [53, 54]. Many of the enzyme molecules present in the lumen of the duodenum do not originate in the duodenal epithelium but in the pancreas (lipases, phospholipases, amylases, trypsin, and several others), but their function nonetheless requires the provision of a privileged aqueous milieu, and therefore a barrier. The blood-intestinal barrier in the jejunum and ileum mediates both uptake and secretion. It regulates uptake via specific transporters located in the apical brush border membrane (for example the (inducible) glucose transporter [55], and the PEPT1 transporter responsible for the uptake of di- and tripeptides formed during the digestion of proteins [56], but also the ABC efflux transporters at the apical membrane of the cells: P-gp, MDR1, MRP2 and also MRP4. The intestinal barrier also allows uptake of proteins from the lumen to allow surveillance of luminal antigens [20, 57]. The brush border of the ileal enterocytes contains enzymes for the final digestion of small molecules: sucrase, lactase, maltase. The mucus overlying the ileal enterocytes, particularly in the terminal region of the ileum, contains a population of commensal bacteria [58] and therefore resembles the mucus overlying the enterocytes in the colon, discussed below.

The blood-intestinal barrier in the colon will be considered here in more detail than the other intestinal barriers. In the colon, the blood-tissue barrier regulates material exchanges between the blood and the fecal compartment. In particular it takes up water and sodium and chloride ions out of the fecal material [59, 60]. The mechanisms taking up water must however exclude many potentially pathogenic cells which are present in large numbers (∼10^13) p in the bowel flora [61]; these should not reach the epithelium and, definitively, should not pass through the colon barriers and enter the blood. The entire colon epithelium therefore expresses molecules such as the antimicrobial peptides β-defensins [20], which contribute to maintenance of the barrier. The barrier is also rendered more effective by intestinal propulsive motility, by cell turnover of the epithelial cells every 4 – 7 days [62], and by rapid repair processes [20]. The colon barrier, with its numerous functional and structural aspects, is large: the movement of water and particular solutes from the lumen to the blood takes place over an area which, in humans, is approximately 0.2 m² [60], or 10 – 20 times larger if the increased area due to crypts is included. The structural basis of the colon barrier is the single-layered colon...
epithelium, which is well-adapted for fluid uptake, with a brush border facilitating absorption; to render it an effective barrier it contains (amongst numerous other mechanisms) the mucus-producing goblet cells. The barrier includes tissue in the lamina propria, lying between the epithelial basement membrane and the endothelial cells in the wall of the nearest blood vessel, and also cell populations residing in the lamina propria. Figure 1 sketches these structures. We focus here on those parts of the barrier formed by the epithelial cells. As is usually the case in mucosal epithelial barriers, the epithelial part of the barrier is double, consisting of a transcellular and a paracellular part. The bodies of the epithelial cells form the transcellular barrier; the paracellular barrier is formed by the tight junctions [6]. The barrier is tighter in the colon than in the small intestine [63]. In addition to these, the two mucus layers overlying the enterocytes represent a further major barrier regulating the transfer of materials and of cells. The outer mucus layer accommodates numerous commensal bacteria [64], as biofilms which displace potentially pathogenic bacteria and other organisms and which promote maintenance of the mucus layers [58, 65]. The mucins are more highly sulphated in the colon than in other intestinal segments, perhaps due to an adaptive response to the commensal bacteria [66]. As part of the barrier function, the mucus contains secreted IgA, and also contains binding sites that compete with the epithelial binding sites [20]. Mucus viscosity increases towards the distal colon, separating bacteria according to their physical properties and position in the colon [66]. Failure of mucus chemistry impairs the barrier [67] and is associated with bowel diseases [68].

The structurally and physiologically complex intestinal barriers, with their multiplex regulatory systems, are essential to health. Even a partial breakdown of intestinal barriers has serious consequences, for example food intolerance, or autoimmune or inflammatory diseases [6, 69]. Indeed, (partial) barrier failure is increasingly considered to be one of the causative factors in an ever wider range of diseases [9]. In this review, however, we focus on intact barriers. Tumors, for example in the form of adenomas, first hide behind the intact barriers (Figure 1), then at more advanced stages they pierce the barriers during the early stages of metastasis, and finally they disrupt the barriers completely. This review focuses on the stages at which the barriers are intact, and describes how intact barriers dose-limit conventional drug therapies, but provide the potential for highly selective targeting in the nanomedicine of the future.

**Small drug molecules cross barriers**

Drugs with a molecular size less than ~ 500 Daltons cross most blood-tissue barriers, providing they can partition between aqueous and lipid phases. When designed to have small or no electrical charge in the physiological pH range, they will pass through the cell membranes and, also, often between the cells in the paracellular pathway. Only the highest barriers (brain, testis, placenta) exclude small drug molecules from entering the organ’s internal fluid compartment. As a result, small molecules enter most organs more or less rapidly, thus distributing into many different functional compartments within the organism. The proportion of drug delivered into the target lesion is reduced by this widespread distribution; if we suppose that (in a “thought experiment”) distribution throughout the body volume were purely diffusive and encountered no barriers, then a 1 g tumor (~ 1 ml in volume) in a 70 kg person (~ 70 l = 70,000 ml in volume) would receive only ~ 1/70,000 (0.0014%) of the total drug dose applied. In real organisms there are numerous reasons why a higher percentage may be achieved, for example the presence of influx pumps at target cell surfaces. Experience has however shown that even drugs which have specific targeting capacities, such as monoclonal antibodies, achieve delivery of only 0.001 – 0.01% of the applied dose into their parenchymal targets after intravenous application [1, 70]. The widespread distribution of small-molecule drugs facilitates the occurrence of side-effects. Steep gradients may be required to drive the drug across the local barrier into the target compartment, necessitating higher total doses of drug in the bloodstream, this in turn raises the concentrations of drug appearing in non-target tissues and thus increases the incidence and severity of side-effects. As a result, therapeutic indices can become narrow [71, 72, 73, 74, 75], culminating in failure to treat the lesion adequately [76]. The resulting balancing act, aiming to find an optimum dose between efficacy and toxicity, particularly in drugs with narrow therapeutic index such as those commonly used in oncology, occasions much discussion at present [77, 78]. Dose-limitation of small-molecule drugs usually results from side-effects arising because small molecules are able to cross many blood-tissue barriers in the body, which enables them to enter numerous non-target sites and there exert effects in non-target tissues.
Macromolecules and nanoparticles do not cross intact barriers

Unless they bear specially selected targeting groups (discussed below), large macromolecules and nanoparticles do not cross cell membranes, and are also too large to pass the paracellular pathway, therefore they do not cross intact blood-tissue barriers. In principle, therefore, they cannot leave the bloodstream, so they do not distribute into all tissue compartments of the body and therefore cannot cause side-effects. At sites where the blood vessels are permeable and barriers are reduced or absent (e.g., in proximity to advanced tumors), macromolecules and nanoparticles wash-in to a lesion rapidly and wash-out from it slowly (enhanced permeability and retention = “EPR”), and this permits, for example, antibodies and targeted nanoparticles to accumulate in close proximity to their target molecules. Except at permeable sites such as the liver, and lesion sites (EPR), nanoparticles therefore generally exit from the bloodstream via the reticuloendothelial system. Since they do not cross intact barriers, a higher concentration of them in the bloodstream will result (due to EPR) in a higher concentration exclusively in the lesion, and nowhere else. This result is exemplified by one of the first nanovectors to enter lesions via EPR and also transport mechanisms, Abraxane®. Its relative lack of side-effects allows a higher dose to accumulate in the tumor, with delivery efficiency reaching ~ 2% [79]. Non-targeted nanoparticles such as liposomes reach delivery efficiencies of 0.3 – 1.3% due to EPR, and also depending on their “stealth” properties [80]. In sum, non-targeted, large macromolecules and nanoparticles do not leave the bloodstream except at sites of EPR – the resulting improvement in drug delivery arises because nanoparticles cannot cross other blood-tissue barriers than those affected by the lesion. However, access to lesions via EPR entails one major consequence: since they do not pass intact barriers, macromolecules and nanoparticles aimed to “target” lesion sites via EPR are not able to eradicate early-stage tumor cells and metastases hiding behind intact blood-tissue barriers. Macromolecules and nanoparticles aimed at crossing intact blood tissue barriers need attachment of special targeting groups for endothelial caveolae and lesion specific antibodies [1].

Lesions behind intact barriers

Early-stage tumors, consisting of only a few cells, hide behind intact blood-tissue barriers, and differ from their nearest neighbors in only a few characteristics (Figure 1). Micrometastases, and a few tumor cells at the outer edge of any large tumor mass, also reside behind the local blood-tissue barrier. These cells cannot be accessed via EPR. In the case of colorectal carcinomas, the local barrier is the complex and tightly regulated blood-colon barrier. On the one hand, cells hidden behind an intact barrier will not be reached by non-targeted nanoparticles, and on the other hand small-molecule drugs will not eradicate all these cells because an adequate dose cannot be built up in the lesion without injecting a total dose that would cause unacceptable toxicity to the host body [76]. The few tumor cells hidden behind such a blood-tissue barrier can survive a small-molecule drug therapy, and then later may generate a metastasis. To eliminate these dangerous cells it is essential to apply drugs by transbarrier transport, whether this be done by transfer of targeted nanoparticles specifically across the local blood-tissue barriers, or by topical application to the luminal side of a mucus barrier. Transbarrier access to hidden tumor cells is necessary to prevent later metastasis. As we have described [1], double-targeting is essential to access cells hiding behind intact blood-tissue barriers, though single-targeting is adequate to access the cells across a mucus barrier.

Transbarrier targeting of nanoparticles

A. Design parameters of nanoparticles for systemic application

Figure 2 shows a sketch of the design requirements for a nanoparticle capable of delivering a drug load (for example, an siRNA payload) to colorectal cancer cells hiding behind the blood-colon barrier. These design requirements are discussed here. “Transbarrier” nanoparticles must be crosslinked, to ensure they do not disintegrate during passage to the target cells. Biocompatible transbarrier nanoparticles, with low toxicity and immunogenicity, can be prepared from natural proteins normally produced in the healthy body, such as HSA [81]. An optimum size exists for the nanoparticles, guaranteeing uptake by physiological mechanisms because the nanoparticles are sufficiently small, yet carrying an adequate drug payload because they are sufficiently large. Nanoparticles of 30 – 50 nm diameter fulfill these requirements, passing readily through endothelial cell caveolae [82] and yet carrying ~20 siRNA molecules in each nanopar-
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In view of the rapid saturation of the target sites, both of the local endothelial targets and also of the disease-specific molecules, only relatively small numbers of nanoparticles can be navigated into the lesion during a period of a few hours [1], so that such nanocarriers should be used to transport maximally potent drug molecules. This means that drugs comprising nucleic acid molecules (such as siRNA) [84] should be incorporated into “transbarrier” nanoparticles, because amplification is an intrinsic part of drug action for these molecules [85]. Furthermore, the extra level of specificity, built into the system by use of highly specific nucleic acids, reduces any potential side-effects because – even if released into the wrong target – these drugs target mRNA molecules in a highly specific fashion, and so will not exert actions in non-target cells. Finally, as seen in Figure 2, the nanoparticles should bear at least two specificities of antibody. The first of these (#1 in Figure 2) should have specificity directed against protein components of one of the major transendothelial transport systems, endothelial caveolae, and in particular caveolae of the endothelial cells in the closest vicinity of the diseased tissue. Specificity #1 should navigate the nanoparticles into the direct vicinity of the target cells, deep within a tissue and behind its barriers, by “flagging and ferrying” mechanisms [1]. The second of the antibody specificities (#2 in Figure 2) should be aimed at characteristic protein features (“biomarkers”) of the cells forming the lesion, and should mediate binding to these cells followed by uptake into these cells. In the case of colorectal cancer, the antibody molecule with specificity #1 should recognize endothelial caveolar components from the microvascular endothelial cells in the colon walls. The antibody with specificity #2 should recognize a specific biomarker for colorectal cancer, for example the receptor for epithelial growth factor (EGF-R). Only a small number of antibody groups need to be attached to a 50 nm diameter nanoparticle: 2 or 3 of each specificity suffice to anchor the nanoparticle to the target cell; this principle is “double targeting for transbarrier passage” [1]. Using metal nanoparticles with appropriate antibodies attached, drug delivery rates of 80 – 90% have been reported [86], the ~8,500-fold improvement over the delivery efficiency of small-molecule drugs depending on targeting of the particle to the local endothelial caveolae. Organ-specific, tissue-specific, and lesion-specific determinants exist in endothelial caveolae, and can be identified by proteomics [87] or by phage-screening techniques [88]. Identifying and targeting such determinants can be seen as a grand challenge in both oncology and in Nanomedicine, but it has been shown that it can be done. Success will open the way to transbarrier treatment of many solid tumors.

B. Design parameters of nanoparticles for topical application

Nanoparticles designed for topical application to mucosal epithelia must pass through the complex mucus layers, retaining their physical integrity during that pas-
sage and arriving at the epithelial cell layer still capable of binding to their target cells specifically. The particles should therefore not have muco-adhesive properties which would prolong their residence time in the mucus layers, and this can be achieved by coating them with neutrally charged polymers such as polyethylene glycol (PEG) [89, 90]. Although the meshwork spacing of mucus would indicate that particles should be less than 55 nanometers in diameter to pass through mucus, it has been reported that neutrally charged PEGylated 500 nm diameter nanoparticles do in fact pass rapidly through human mucus [91]. Since the size of nanoparticles is therefore not constrained by the need to pass through the mucus, then the proportions we discussed earlier [1] can be used. Thus, nanoparticles for transbarrier passage through mucus layers can be 30 – 50 nm in diameter, PEGylated, and bearing 1 – 3 targeting groups directed to bind specifically to the biomarker molecules (such as EGFR) present on the malignantly transformed tumor cells. Since topical application is carried out at the apical aspect of the intestinal epithelium, no targeting of endothelial proteins is necessary, and the particle can therefore be prepared without the antibody #1 attached to it, though the antibody #2 is still required (compare Figure 2). To avoid digestion of the nanoparticles by (for example, digestive) enzymes as they pass through the mucus layers, PEGylation with PEG chains of appropriate size prevents access of the enzymes to the particles [92]; this is necessary in mucus of the small intestine, but is not needed when targeting the colon epithelium because this lacks digestive enzymes in its mucus layer [93]. By appropriate formulation of gastrointestinal capsule packaging, topical application can be modified for oral application.

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A review of bidirectional transbarrier targeting in the intestine and options that exist for oncology.


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