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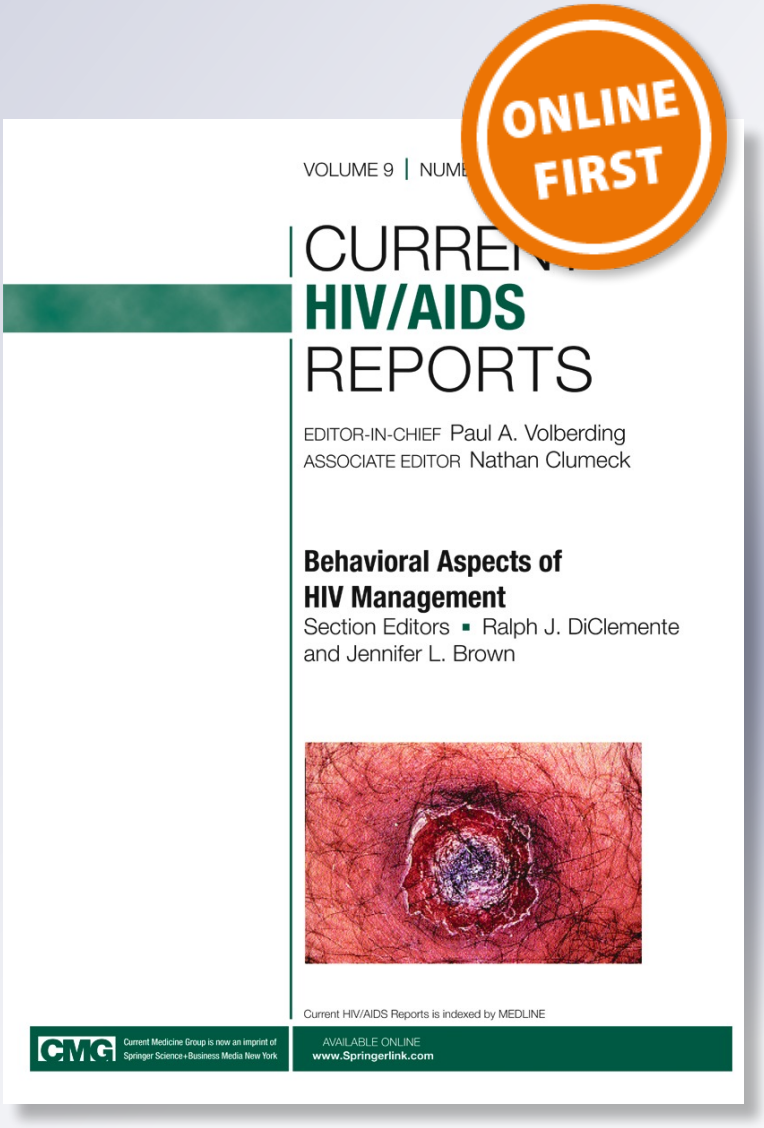

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HIV PATHOGENESIS AND TREATMENT (A LANDAY, SECTION EDITOR)

Use of Human Mucosal Tissue to Study HIV-1 Pathogenesis and Evaluate HIV-1 Prevention Modalities

Charlene S. Dezzutti · Florian Hladik

Abstract The use of human mucosal tissue models is an important tool advancing our understanding of the specific mechanisms of sexual HIV transmission. Despite 30 years of study, major gaps remain, including how HIV-1 transverses the epithelium and the identity of the early immune targets (gate keepers). Because defining HIV-1 transmission in vivo is difficult, mucosal tissue is being used ex vivo to identify key steps in HIV-1 entry and early dissemination. Elucidating early events of HIV-1 infection will help us develop more potent and specific HIV-1 preventatives such as microbicides and vaccines. Mucosal tissue has been incorporated into testing regimens for antiretroviral drugs and monoclonal antibodies. The use of mucosal tissue recapitulates the epithelium and immune cells that would be exposed in vivo to virus and drug. This review will discuss the use of mucosal tissue to better understand HIV-1 pathogenesis and prevention modalities.

Keywords Mucosal tissue · Sexual transmission · Sexual HIV transmission · HIV-1 infection · HIV pathogenesis · Microbicide · Vaccine · Explants · Biopsies

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Introduction

While the use of macaque models to study the early events associated with SIV/SHIV transmission has been informative in identifying early reservoirs and viral dissemination [1, 2], there are differences between humans and macaques as well as SIV and HIV-1. Consequently, gaps remain in our knowledge and assumptions have to be made to extrapolate the findings to human transmission. Some researchers use man-made organotypic models to represent human mucosa, but these models lack the full epithelial thickness (up to 30 layers thick for vaginal/ectocervical epithelium) and the correct type and proportion of mucosal immune cells [3]. To address some of these concerns, researchers have turned to human mucosal tissue to study these early events ex vivo. The primary focus has been on vaginal and ectocervical tissue acquired from surgical procedures. Colorectal, foreskin, and penile tissues have been used as well. Ex vivo human tissue provides the correct architecture and immune targets for HIV-1 infection and product evaluation that other models cannot duplicate. The data gathered through this work is helping to define how HIV-1 transverses the epithelium, infects its target cells and disseminates, as well as how to effectively design and test microbicides and vaccines to prevent infection. This information should reveal opportunities to refine and further develop HIV-1 preventatives, such as topical microbicides and vaccines. This review discusses the contribution of ex vivo mucosal tissue to our understanding of HIV-1 pathogenesis and defining safe and effective HIV-1 preventatives.

Use of Mucosal Tissue to Study HIV-1 Pathogenesis

In the early 1990s, about ten years after the identification of HIV-1 as the causative agent of AIDS, it became clear that

finding an efficacious preventative vaccine would likely be more difficult than expected [4]. HIV-1 vaccine candidates at the time had failed to induce high-titer cross-neutralizing antibodies and cytotoxic T cell responses, and the extent of viral spread throughout the lymphoid system early during infection was recognized as a difficult problem in developing effective treatments and a protective vaccine [5, 6]. Consequently, researchers turned their focus to the mechanisms of HIV-1 transmission across mucosal surfaces and subsequent viral spread and amplification in lymphatic tissues. It was hoped that these studies would provide clues as to how a vaccine or other emerging prevention technologies such as topical microbicides should be designed to effectively protect mucosal surfaces from HIV-1 infection.

Some important early findings were made in human skin models demonstrating that cutaneous dendritic cells (DC) were potent facilitators of productive HIV-1 infection [7, 8]. Limitations of these early models of HIV-1 pathogenesis were the use of skin in the place of the less accessible genital mucosa and the strategy of isolating cells from the tissue before performing infection studies. When the first report of HIV-1 infection in DCs from the female genital mucosa was published, the former limitation was overcome but the work was still done with cells isolated from the mucosa rather than in situ [9]. Extracting cells from tissue, either with enzymes or by allowing migratory cells to actively exit from the mucosa can lead to changes in cell activation and surface receptor expression that may alter their interaction with HIV-1 [10, 11].

Then, in 2000, came two prominent studies that investigated HIV-1 infection within intact cervical explants containing both epithelium and stroma, one by Gupta and colleagues [12] and the other by Shattock and colleagues [13]. The Shattock model was based on earlier work published by the same group in 1994 [14]. In these studies, the researchers inoculated the tissue with HIV-1 and then stained sections by immunohistochemistry for HIV-1 gag protein or by in situ hybridization for HIV-1 RNA. HIV-1 infected cells were noted immediately beneath the epithelial layer and were identified mostly as $CD4^+$ T cells in the Gupta model [12] and as $CD68^+$ macrophages in the Shattock model [13]. Studies since then have confirmed the susceptibility of both cell types in the female genital mucosa to HIV-1 infection [15–17, 18••, 19–21], so the results of the two early models appear to be correct, with the discrepancy in targeted cells types likely attributable to technical variations and sensitivities of the assays used.

One major difference between the techniques was that the Gupta model was the first claiming to be "polarized", meaning that it mimicked the route of natural infection in vivo where HIV-1 has to penetrate the epithelial surface to reach leukocytes embedded deeper within the epithelium or the stroma. Polarity was achieved by using agarose to seal tissue plugs placed onto transwell filter inserts, forcing the virus to enter the mucosa from the surface oriented in vivo toward the vaginal cavity, whereas media nurturing the tissue was supplied from the bottom chamber of the transwell through the filter membrane. While there has been controversy over whether this initial model was truly polarized, the Gupta group as well as others subsequently improved upon it, so that newer studies with cervicovaginal, foreskin, or colorectal explants can make this claim more convincingly [16, 21–25].

In what scenarios is explant polarity important? For many pathogenesis studies of HIV-1 infection, it is in fact secondary. While it would be nice to be exact in recapitulating the directionality of mucosal HIV-1 infection in vivo, the events following contact between an infectious virion and a susceptible target cell are unlikely to be altered by how HIV-1 reached the cell in the first place. For this reason, most pathogenesis studies [19, 26, 27••], have not used polarized models [17, 18••, 28, 29]. Conclusions about which cells in the cervicovaginal mucosa are most susceptible to HIV-1 entry and infection, how the virus penetrates the outer cell membrane, its intracellular fate, and its route of cell-to-cell spread can be reached without simulating tissue polarity.

In this way, it has been demonstrated that $CD4^+$ T memory cells are the main drivers of productive infection in the cervicovaginal mucosa [16, 17, 18••, 19]. Other studies also have identified productive infection of $CCR5^+$ macrophages [13, 15, 21]. How HIV-1 reaches these target cells is not clear, but DCs are likely to play a role. HIV-1 can enter DCs not only via CD4 and CCR5 but also through C-type lectin receptors such as DC-SIGN and langerin [30, 31]. The dendrites of DCs form a network within the squamous epithelia, such as those covering the vagina and ectocervix, which reach higher toward the luminal surface than T cells [32–37]. In columnar epithelia, DCs can stretch their dendrites through epithelial tight junctions and can sample the luminal contents [38, 39]. Thus, it is plausible to assume that they are frequently the first leukocyte type encountered by HIV-1 in the mucosa.

When HIV-1 reaches $CD1a^+$ DCs termed Langerhans cells (LCs) residing within the squamous epithelium of the vagina and ectocervix, the LCs endocytose virions very efficiently [19] and, without themselves being productively infected, pass HIV-1 to susceptible $CD4^+$ T cells [27••]. For most of these studies, vaginal epithelial sheets were isolated by ex vivo suction blistering. Based on experiences with human skin [40–42], a customized suction cup apparatus applied for ~90 minutes gently creates 5 mm diameter blisters whose roofs are formed by the mucosal epithelial layer. The epithelial-stromal separation takes place at the lamina lucida of the basal membrane–the blister roof can then be excised to yield intact epithelial sheets untouched by digestive enzymes, ensuring that HIV-1 receptors on the cell surface remain unaltered.

Though it's clear that vaginal LCs can transport infectious HIV-1 this does not mean they are necessarily the first cell type to capture HIV-1 in the cervicovaginal mucosa. The sequence of events unfolding when HIV-1 penetrates the mucosa can only be specified by forcing the virus through the initial steps of transmission in the same directionality as they occur in vivo, necessitating a polarized tissue model. Likewise, studying how HIV-1 breaches the intact outer epithelial barrier of the cervix or vagina, as well as the mucus film covering it, requires polarized explants. These are difficult experiments. Even if a large amount of fluorescently labeled HIV-1 is applied to the mucosal surface, the number of observable virions decreases exponentially with the depth of penetration into the epithelium, making a clear distinction between background auto fluorescent debris and virions increasingly difficult. This problem can be overcome by using virions tagged with a green fluorescent protein-viral protein R (GFP-Vpr) fusion protein where the GFP molecule is photo-activatable [43]. Any dots not increasing dramatically in fluorescence upon photo-activation are revealed as background, and the certainty of virion identification can be further increased by co-labeling virions with anti-gag antibodies or lipid membrane dyes [44]. Using this technique, T. Hope and colleagues have presented results at scientific conferences showing that HIV-1 is able to move through the interstitial spaces between differentiated squamous epithelial cells of the ectocervix to depths of 40 μm, well within reach of embedded LCs.

This unimpeded passage is supported by a recent finding that the apical layers of the ectocervical and vaginal epithelium do not contain classical cell-cell tight junctions and are permeable to IgG [45•]. In contrast, endocervical columnar cells are joined by IgG-impermeable tight junctions. It has been generally assumed that the single layer columnar epithelium of the endocervix is the site most vulnerable to HIV-1 invasion. However, these results suggest that the vagina and ectocervix may be more vulnerable, even without micro-abrasions, and that LC dendrites reaching into the tight junction-free zone of the epithelium could be the bridge to transport HIV-1 deeper into the epithelium where activated $CD4^+$ $CCR5^+$ T cells reside. This principle has recently been affirmed by HIV-1 infection studies with polarized inner human foreskin explants [22, 46]. Interestingly, LCs in the uppermost layers of the vaginal epithelium do not express langerin [27••], which can target HIV-1 to an intracellular degradation pathway [47]. Thus, the combination of a tight junction-free superficial epithelium with langerin-negative LCs concentrated in that area could be exploited by HIV-1 to establish infection in the vagina and ectocervix. Confirming this hypothesis for the cervicovaginal mucosa will require HIV-1/langerin/CD1a co-localization studies in polarized cervicovaginal explants.

Lastly, polarized mucosal explants provide a tool to investigate mechanisms of viral selection. It is well known that among two broad categories of HIV-1 variants, those using CCR5 and those using CXCR4 as co-receptors for fusion with the cell membrane, transmissions occur nearly exclusively with HIV-1 using CCR5 [48]. However, why this occurs remains unclear, with the current hypothesis being a plausible but still vague multiple gatekeeper concept; imperfect selection occurs sequentially at multiple levels, cumulatively giving HIV-1/CCR5 a strong advantage [49•]. One study using a non-polarized explant model indicated that HIV-1/CCR5 replicates more efficiently than HIV-1/CXCR4 in cervicovaginal tissue [18••]. Studies in a polarized model could extend these findings to include additional possible levels of gate keeping, in particular differential interaction of HIV-1 with epithelial cells and LCs during viral penetration into the mucosa. For example, expression of CXCR4 but not CCR5 on cervical epithelial cells has been observed (Hladik unpublished data and [50, 51]) and could play a role in sequestering HIV-1/CXCR4. Thus, viral selection and comparative fitness studies are best performed in polarized models [52], as these models encompass several levels of potential restriction and represent the overall capacity of an HIV-1 variant to overcome these barriers better than a non-polarized model.

Use of Mucosal Tissue to Evaluate HIV-1 Prevention Modalities

Preclinical efficacy and safety testing of microbicide products and antibodies has been done using a variety of in vitro assays that use primary immune cells and molecular indicator cell lines. There are several algorithms in use that vary in the specific assays performed due to laboratory preferences [53–56]. These assays characterize the performance of the drugs or antibodies under different environmental conditions (pH transition), against drug resistant HIV-1, and/or cell-associated HIV-1. Until this past decade, most algorithms have not incorporated ex vivo mucosal tissue which includes cervical, vaginal, colonic, penile, foreskin, and tonsil. Both polarized and non-polarized systems are used to address specific questions regarding product safety, efficacy, and drug localization. To note, colorectal and tonsil tissue are traditionally placed on gel-foam rafts, regardless of polarization, to provide 3-dimensional support for the tissue [24, 57, 58].

Non-polarized tissue is typically composed of small cubes (2 or 3 mm^3) of tissue that are cut from the larger piece using a scalpel. The epithelium is retained, but the muscularis is trimmed off [13, 57, 59, 60]. Alternatively, the stratified squamous epithelium can be separated from the underlying stroma after treating the entire tissue with EDTA

overnight, resulting in intact vaginal epithelial sheets [61]. The tissue or sheet is submerged in medium that contains drug with or without HIV-1. This creates the "worst-case scenario" by allowing virus access to targets in the lamina propria independent of traversing the epithelium. Non-polarized models have the advantage of more efficiently utilizing the available tissue. Consequently, many tissue replicates are possible for each treatment condition. Using this model system, drug entities known as active pharmaceutical ingredients (APIs) have been tested to determine safety in tissue and ability to prevent HIV-1 and dissemination [13, 59, 62–66, 67••, 68–72] as well as HSV-2 infection [73•, 74] (Table 1). Non-polarized tissue is pre-treated with the API for a short period of time, typically 15 to 30 minutes, and then virus is added to the cultures. After 2 hours, the tissue is washed and placed in fresh medium. The potency of several entry inhibitors and non-nucleoside and nucleotide reverse transcriptase inhibitors has been tested in non-polarized tissue demonstrating that several $\log_{10}$ more drug is needed to block HIV-1 infection of tissue than what is found for traditional in vitro assays such as indicator cell lines [59, 63, 67••, 68]. This has created the possibility of defining the effective dose that will block HIV-1 infection by 50 % to 100 % in the tissue–in essence creating a "tissue concentration effective dose 50 % or 100 %". These models also demonstrated the benefits of drug combinations, which much like therapy, show an additive effect even in the presence of drug-resistant virus [67••, 68]. An additional utilization of the non-polarized tissue models has been evaluating the ability of drug to block HIV-1 infection of the cells that migrate out from the tissue thereby stopping dissemination of the virus [17, 59, 63, 64].

Evaluation of formulated API(s), whether they be aqueous gels, films, creams, tablets, or other dosage forms, is typically done using polarized tissue because the use of the topical product is intended to primarily interact with the epithelium. The tissue is oriented with the apical surface upward and sealed around the sides using agarose or Matrigel™. Most microbicides to date have been formulated as aqueous-based gels [21, 24, 75–80, 81•, 82] (Table 1). Preclinical testing of the gels adds additional complexity because pH, osmolality, and viscosity of the product will impact the results. For instance, the polymers used in the formulation may alter the overall toxicity or efficacy profile due to smothering of individual cells or non-specifically binding HIV-1. Tissue-based screening is advantageous because effects of the vehicle independent of the API can be evaluated to define the impact the formulation may have on the testing results. As new formulations are designed, researchers need to become more creative with their testing. For example, solid dosage forms using quick-dissolving film technologies are first allowed to dissolute in a small volume of liquid before being added to the apical tissue surface. Testing these films has shown them to safely and effectively deliver RC-101 [79] and dapivirine [83] to cervical tissue. Using a more novel formulation approach, researchers have created subliming matrices to deliver any drug of interest thus creating a "universal" delivery system. The solids are applied to the apical surface and while they sublime away, drug is delivered to the tissue. Because delivery occurs over several days, the tissue can be challenged with HIV-1 over this time period. This approach successfully tested the delivery of tenofovir and emtricitabine to polarized cervical tissue exposed repeatedly to HIV-1 and prevented infection of the tissue [84••].

For topical microbicides, the original paradigm was the development of vaginal products for women to protect themselves against HIV-1 acquisition. However, it was realized that products marketed for vaginal use would be used by all persons wanting to protect themselves including those engaging in receptive anal intercourse. With this in mind, non-polarized [57] and polarized [24] colorectal tissue models were developed for screening the safety and efficacy of vaginal microbicide products specifically for this compartment. Several APIs have been tested and the relative efficacy in the tissue with non-nucleoside reverse transcriptase inhibitors showing more potent activity than nucleotide reverse transcriptase inhibitors and entry/fusion inhibitors [66, 67••, 68]. While the APIs have shown no detrimental toxicity to the mucosal tissue, formulations of several vaginal microbicide candidates showed loss of cervical and colorectal epithelium [21, 24, 80]. This could be attributed to the concentration of the API in the formulation, as was the case for PRO 2000 where the 2 %, but not the 0.5 %, concentration showed tissue toxicity [21, 24]. Or it could be attributed to the formulation itself. The original tenofovir 1 % gel is hyperosmolar and the active and vehicle control gels both showed epithelial sloughing and fracture [80]. When reformulated to lower the glycerin content and thus reducing the osmolality, the new tenofovir 1 % gel showed no epithelial changes [77]. This was reflected in the clinical trials using the original tenofovir gel for rectal application; several participants experienced cramping, abdominal bloating, and urgency–typical of hyperosmolar products such as enemas [85]. When the reformulated tenofovir gel was applied rectally, it was better tolerated than the original formulation [86]. There is now a concerted effort to develop microbicides specifically designed for use during receptive anal intercourse [81•].

To begin to bring real life experiences into the laboratory, mucosal secretions are being added to mucosal tissue to study their effects on drug activity. Semen or seminal fluid has been the most studied secretion and reduces the potency of several microbicide polyanion drugs such as PRO 2000 and VivaGel [87, 88] while not impacting non-nucleoside and nucleotide reverse transcriptase inhibitors [78, 89].

Table 1 Use of ex vivo human mucosal tissue to evaluate HIV-1 topical microbicides and antibodies using non-polarized and polarized models

Model	Tissue type	Drug (formulation)	Reference
Non-polarized tissue	Cervix	PRO 2000, UC781, PPCM, PMPA, dapivirine	[13, 63–65, 72]
	Cervix	nAb, B12 & CD4-IgG2	[17]
	Cervix	Cellulose acetate phthalate gel	[69]
	Cervix	Griffithsin	[62, 70]
	Vaginal sheets	Cellulose sulfate gel, T20	[61]
	Penile	Cyanovirin-N & PRO 2000	[59]
	Colorectal	UC781, Dapivirine, PMPA, emtricitabine	[67••, 68]
	Colorectal	L'644, C34, T20, T1249	[66]
	Tonsil	AOP-RANTES, Cellulose acetate phthalate gel, acyclovir, tenofovir	[71, 73•, 74]
Polarized tissue	Cervix	RC-101 (aqueous gel & film)	[79, 82]
	Cervix	Tenofovir/UC781 combination (aqueous gel)	[76]
	Cervix	Tenofovir & emtricitabine (subliming solid matrices)	[84••]
	Cervix	Dapivirine (Film)	[83]
	Cervix/colorectal	Cellulose acetate phthalate, Carraguard™, D2A21, PRO 2000, UC781, VivaGel™ (aqueous gels)	[21, 24]
	Cervix/Colorectal	IQP-0528 & IQP-0528/tenofovir combination (aqueous gels)	[75, 78]
	Cervix/Colorectal	Tenofovir (aqueous gel & reformulated aqueous gel)	[77, 80]
	Colorectal	Rectal-specific placebos (gels)	[81•]
Ex vivo challenge–non-polarized	Colorectal biopsies	UC781 gel–rectal use	[95••]
	Colorectal biopsies	Tenofovir 1 % gel–rectal use	[85]

While semen was purported to enhance HIV-1 replication in tonsil tissue [90], its ability to enhance HIV-1 replication in cervical and colonic tissue has not been demonstrated yet (Dezzutti unpublished data). Incorporating female genital tract fluid can be more problematic. If a cervicovaginal lavage is used, the genital tract fluids are diluted 30- to 40-fold, thus limiting the usefulness of this approach for testing drug activity. Likewise, undiluted female genital tract fluid collected by direct aspirate or the Instead Cup could be incorporated, but there are concerns about its usefulness as well for several reasons: (i) volumes of material collected are typically <1 ml making it difficult to use for multiple assays, (ii) immune factors vary across the menstrual cycle, and (iii) high levels of genital tract flora will be problematic in mammalian tissue culture. However, as progress is made, research groups will begin to incorporate mucosal secretions into their testing algorithms to recapitulate the environment in which HIV-1 is transmitted.

The use of mucosal tissue to date has not only provided critical information regarding the feasibility of using antiretroviral drugs for HIV-1 prevention, but also demonstrated some predictability of clinical success. Preclinical testing, which included tissue explant testing, of products used in phase 3 efficacy trials (Carraguard [91], nonoxynol-9 [92], and cellulose sulfate [93]) showed these compounds either (i) were not effective against HIV-1 infection [21, 54] and resulted in no benefit for the trial participants or (ii) were

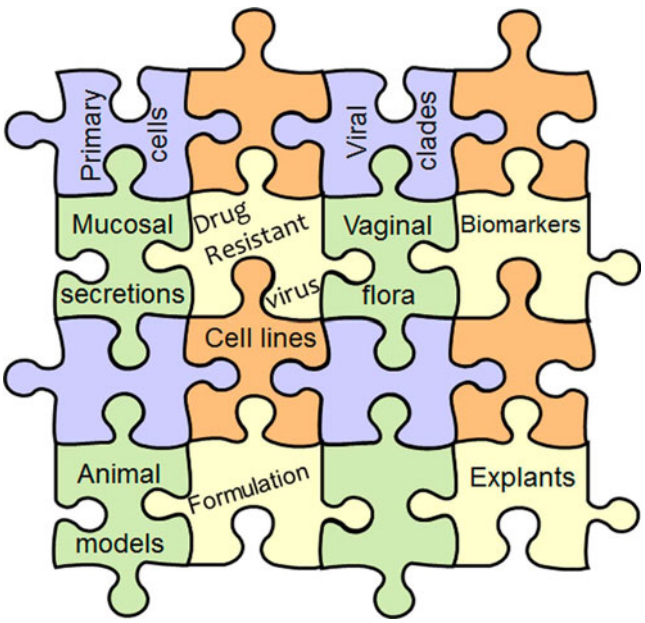

Fig. 1 The HIV-1 pathogenesis and preclinical testing puzzle. Human mucosal tissue when used ex vivo is termed explants and is one of the corner stones of laboratory testing. The advantage of mucosal ex vivo tissue is it provides the anatomical site where virus enters the host and topical microbicides and antibodies encounter it. To fully address HIV-1 pathogenesis questions as well as the safety and efficacy of HIV-1 preventatives, mucosal ex vivo tissue should be used in conjunction with other models and modifiers

toxic [21, 24, 63, 94] and thus led to increased HIV-1 infection in the clinical trial. A new approach to evaluating topical microbicide products has been the incorporation of ex vivo mucosal tissue in early phase 1 safety trials. Termed "ex vivo challenge" assays, participants use a product daily for up to 7 or 28 days and tissue biopsies are taken after the last dose. The biopsies are placed in culture with HIV-1 in the laboratory, washed, and followed for 14 to 21 days to determine if virus replicates in the tissue or not. Using this approach, rectal use of vaginal microbicide gels containing UC781 [95••] and tenofovir [85] have shown significant protection against HIV-1 infection of colonic tissue. These data are the first to provide a linkage between drug levels (pharmacokinetics) and drug activity (pharmacodynamics). The use of ex vivo challenge assays after vaginal dosing of topical microbicide products has been more challenging. Currently, it is not clear whether vaginal or ectocervical tissue is better for testing. Preliminary work has shown while both tissue types are infected with HIV-1, virus replicates to significantly higher levels in ectocervical tissue [96]. The use of ex vivo challenge using ectocervical tissue is being tested in a phase 1 microbicide trial and the results should be forth coming in the next year [97].

Conclusion

The use of ex vivo mucosal tissue to investigate questions surrounding HIV-1 pathogenesis and the potential of HIV-1 prevention modalities has expanded over the past decade. While results are being generated regarding HIV-1 passage through the epithelium, initial cellular targets, and effective drugs and antibodies that prevent HIV-1 infection there are several limitations for the use of ex vivo mucosal tissue in research. Once removed from the person, the tissue loses hormonal control (especially for cervical tissue), mucus and flora, and the capacity to recruit immune cells. Moreover, after ~36 hours, cervical tissue begins to shed the upper layers of epithelium [12, 13, 21], while colorectal tissue begins to undergo autolysis [24, 57]. Consequently, the functional work done with ex vivo tissue needs to be completed in a short period of time. Despite these challenges, ex vivo explant tissue provides an important piece of the HIV-1 laboratory testing puzzle (Fig. 1) and should ideally be used in conjunction with other models such as organotypic tissue and animal models to fully address the gaps. Ex vivo mucosal tissue, serving as a surrogate for the in vivo environment, remains a critical tool for teasing out the mechanisms of HIV-1 transmission and should continue to be used for the screening of topical microbicides and pathogen-specific antibodies.

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- • Of importance
- •• Of major importance

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