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Review

Reviewing and reconsidering invasion assays in head and neck cancer

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SUMMARY

Head and neck squamous cell carcinomas (HNSCC) are malignant tumors that arise from the surface epithelium of the oral cavity, oropharynx and larynx, primarily due to exposure to chemical carcinogens or the human papilloma virus. Due to their location, dental practitioners are well-positioned to detect the lesions. Deadlier than lymphoma or melanoma, HNSCC is incompletely understood. For these reasons, dental practitioners and researchers are focused on understanding HNSCC and the processes driving it. One of these critical processes is invasion, the degradation of the basement membrane by HNSCC cells with subsequent movement into the underlying connective tissue, blood vessels or nerves. Cancer cells metastasize to distant sites via the blood vessels, lymphatics and nerves. Metastasis is associated with poor survival. Since invasion is essential for development and metastasis of HNSCC, it is essential to understand the mechanism(s) driving this process. Elucidation of the mechanisms involved will facilitate the development of targeted treatment, thereby accelerating development of precision/personalized medicine to treat HNSCC. Robust in vitro and in vivo assays are required to investigate the mechanistic basis of invasion. This review will focus on in vitro and in vivo assays used to study invasion in HNSCC, with special emphasis on some of the latest assays to study HNSCC.

Introduction

Head and neck squamous cell carcinomas (HNSCC) are malignant neoplasms arising from surface epithelium of the oral cavity, oropharynx and larynx. HNSCC may be due to human papilloma virus (HPV) or tobacco and alcohol exposure [1]. Genetic factors and patient behavior are also relevant to development and prognosis [1]. The American Cancer Society estimated that HNSCC accounted for 39,000 new cases and 8000 deaths in the United States in 2011 [2]. Globally, ~600,000 new cases of HNSCC are diagnosed each year [1]. At ~50%, the five-year survival rate is lower than breast cancer or melanoma [3].

HNSCC tumors are heavily vascularized and metastasize through blood vessels, nerves or lymphatic vessels [4] to regional lymph nodes, lungs, bones and the liver [5,6]. Treatment of metastatic HNSCC has poor success even if aggressive, whereas patients with non-metastatic cancer are treated more effectively [5]. Metastases and related complications are leading sources of cancer-related mortality and morbidity [7]. Since metastasis is distant invasion [8], it is critical to understand the mechanisms of invasion. The review highlights approaches used to study invasion, including recently developed in vitro and in vivo invasion assays.

Understanding HNSCC

Fig. 1 provides a graphical illustration of HNSCC. HNSCC develops when genetic abnormalities accumulate in non-malignant epithelial cells. Abnormal cells initially cluster within the surface epithelium (pre-cancer or epithelial dysplasia/carcinoma-in-situ) above the basement membrane. Pre-cancer progresses to HNSCC when the basement membrane is disrupted and transformed cells from the surface epithelium invade the underlying connective tissue [9]. Exposure to chemical carcinogens causes the accumulation of genetic abnormalities, which can result in cancer developing [4]. Multiple primary tumors or recurrent tumors may develop in oral tissues exposed to chemical carcinogens, a phenomenon referred to as "field cancerization" [4].

Metastases of HNSCC may originate from a small population of primary tumor cells [10]. Epithelial to mesenchymal transition (EMT) occurs during HNSCC progression and is characterized by the transition of non-motile epithelial cells into motile mesenchymal-like cells [9]. EMT occurs in wound healing, embryonic development and cancer [9]. In HNSCC, EMT facilitates tumor

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progression, including invasion and metastasis, and potentially increases the population of cancer stem cells [9,11]. After EMT, cells become more capable of initiation of the "invasion–metastasis cascade" [10,12].

Understanding invasion

The terms invasion and migration are often used interchangeably. However, in experimental cellular biology, invasion is defined as disruptive, proteolytic, cellular movement through three-dimensional (3D) barriers, distinct from migration which is defined as non-disruptive, non-proteolytic, movement of cells through tissues [11,13]. "Migration" can also refer to two-dimensional directed movement on a substrate [13].

Despite intense study, the complex and heterogenous mechanisms of invasion and metastasis remain imperfectly understood [14]. Collective or individual invasion of cancer cells occurs via several mechanisms [15,16]. Cells invading collectively exhibit preserved inter-cellular junctions with leader cells paving the way for the collective. In individual cell invasion, each cell invades the basement membrane via highly proteolytic mesenchymal cell movement or plasma membrane blebbing. In HNSCC, disruption of the basement membrane usually occurs via proteolytic degradation of extracellular matrix using matrix metalloproteases (MMPs) [16,17]. Plasma membrane blebbing uses mechanical means to deform and penetrate the basement membrane [15]. Cancer cells may switch from a proteolytic to a mechanical invasion mechanism if matrix degradation by MMPs has been inhibited through protease inhibition [13,15,16]. These invasion patterns have been observed in cancer, wound healing and development [11].

Invasion is essential to metastasis in HNSCC. Invasion and metastasis require penetration of the basement membrane of the surface epithelium and blood vessels to access tissues at proximal and distant sites [8]. Since metastasis is a leading cause of cancer-related mortality and morbidity [7], targeting invasion should slow or prevent metastasis and improve survival in HNSCC.

Invasion assays

Many invasion assays are used, some adapted from migration assays. In vitro invasion assays quantify cells invading through structures such as synthetic Basement Membrane Equivalents (BMEs). The category of BME includes a wide range of materials intended to mimic the structure and composition of the extracellular matrix, from reconstituted collagen gels to more complex materials. These in vitro assays may encourage invasion via chemotaxis, which is the process of cell movement in response to chemical gradients (towards an attractant or away from a repellant). Therefore, assays using chemical gradients can be referred to as chemotaxis-based invasion assays. In vivo invasion assays quantify cells invading through biological membranes that are analogous to the basement membrane.

Since invasion through the basement membrane of the surface epithelium transforms pre-cancer to HNSCC, understanding the mechanism of invasion will identify treatment targets to prevent malignant transformation [9,18]. Unfortunately, there are gaps in traditional experimental models. For example, in vitro assays do not reproduce the complexity of invasion in living tissues, such as the heterogeneity of the extracellular matrix. Moreover, in vivo tumor studies typically feature injection of tumor cells into subcutaneous tissues, which circumvents penetration of the basement membrane of the surface epithelium, preventing investigations of this early event in transformation of pre-cancer to HNSCC [9,18]. Therefore, there is an imperative to develop new and improved invasion assays and understand the traditional assays.

Materials

Due to easy availability, the earliest invasion studies used in vivo or organotypic models such as rabbit ear or amniotic tissue. Difficulties associated with using these materials and quantifying the data produced by these models lead to the development of in vitro alternatives, or BMEs [19]. BMEs include reconstituted collagen with supplements, from sources including rat-tail collagen [17,20]. Reconstituted collagen BMEs require multiple steps to purify and monomerize collagen from an animal, with more extensively cross-linked collagens requiring more extensive processing before being re-polymerized under basic conditions [17]. Matrigel™, produced from secretions of Engelbreth-Holm-Swarm mouse sarcomas, is highly uniform and likely the most widely used BME [20,21]. Matrigel™ contains a range of inherent growth factors, which could potentially confound experiments. Some Matrigel™ advocates argue that this is an advantage rather than a problem because normal in vivo extracellular matrix can also contain growth factors [21,22]. Given the tendency of extracellular matrix to contain bound cytokines, including growth factors, this opinion seems defensible. However, companies provide growth factor-depleted Matrigel™ to avoid these potential problems [21]. Other, less commonly used, BMEs exist and are typically generated from cellular secretions or reconstituted extracellular matrix [21,23]. Many BMEs, containing a range of proteins and other materials normally found in the extracellular matrix, are more structurally heterogenous than simple reconstituted collagen. However, since collagen makes up a large percentage of the extracellular matrix proteins and because it has a crucial structural role, any material that is intended to mimic the extracellular matrix should contain some collagen.

The complexities that made early assays difficult to quantify have major roles in invasion in vivo [24]. This is particularly true of pores in commonly used BMEs, which are more uniform than

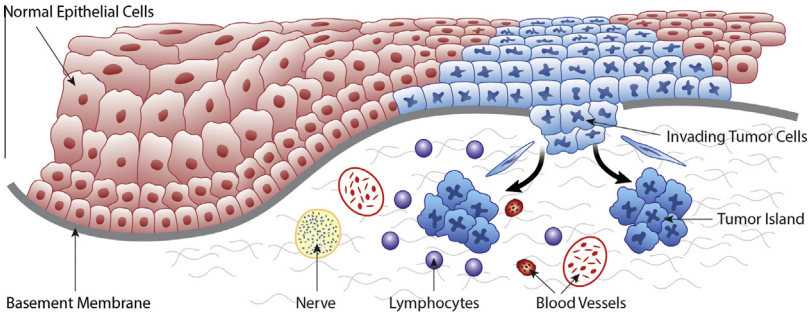

Figure 1. HNSCC invasion. In this image, transformed cells are invading through the basement membrane. Elongated mesenchymal cells represent the importance of EMT in invasion. Invasive tumor islands are present in the connective tissue. Elements of the tumor microenvironment are depicted, including nerves, vasculature and the inflammatory infiltrate. (A color version of this figure is available in the web version of this article).