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Redirecting the focus of cancer immunotherapy to premalignant conditions

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Abstract

Much progress has been made in introducing immunological treatment approaches for cancer, with lessons learned from both the successes and failures of immunotherapy. Among the challenges of immunotherapeutic approaches for cancer are the multitudes of mechanisms by which cancers are known to subvert the immune defenses. This has led to the incorporation into the immunotherapeutic arsenal strategies by which to overcome the cancer's immunological blockades. What has been only superficially explored is the immunological milieu of premalignant lesions and the possibility of immunological approaches for the treatment of premalignant lesions so as to prevent secondary premalignant lesions and their progression to cancer. This review discusses the immunological environment associated with premalignant lesions, and the possible missed opportunity of utilizing immunological treatment strategies in the less hostile environment of premalignant lesions as compared to the immune subversive cancer environment.

Keywords

Cancer; Immune infiltrate; Immunotherapy; Premalignant

Introduction

The survival rates for patients with cancer have been steadily improving with the introduction of a broader armament of treatment options. In addition to surgery, chemotherapy and radiotherapy, several different immunotherapeutic approaches are being used. Such treatments include antibodies such as trastuzumab targeting HER2, cetuximab targeting EGFR or bevacizumab targeting VEGF [1–4]. Non-specific immune-augmenting cytokine treatments such as with IL-2 or IFN-α have been FDA approved and others are in clinical trials [5–7]. Tumor-specific immune treatments have also been tested both alone and in combination with other treatment approaches. These include peptide vaccines and cell-based tumor vaccines [8–10]. While a number of these immunotherapies have seen

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successes in the treatment of cancer patients, common obstacles to immunological cancer treatment are the multitudes of immune subversive and immune evading approaches that cancers possess.

The mechanisms that cancers have to avoid immune defenses are both direct and indirect. Cancers can directly inhibit immune reactivity by secreting soluble immune inhibitory mediators such as PGE_2, TGF-β and IL-10 [11–13]. They also express checkpoint inhibitory ligands such as PD-L1 that block immune reactivity [14]. Indirect immune inhibition by cancers is mediated by their induction of host immune inhibitory cell populations. These include macrophages, Treg cells, Th2 skewed T-cells, myeloid-derived suppressor cells (MDSC) and the less mature $CD34^+$ progenitor cells [15–19]. Within the tumor milieu, there are not only inhibitory immune cell populations, but also immune inhibitory endothelial cells and fibroblasts [20,21]. Some of these immune inhibitory mechanisms can readily be overcome such as by treatment with COX-2 inhibitors to overcome the immune suppressive activity of PGE_2 [15,22]. Treatment with antibodies to checkpoint inhibitory proteins can also overcome a suppressive mechanism [23,24]. This has included clinical use of antibodies targeting checkpoint inhibitory proteins such as CTLA-4, PD-1 and its ligand PD-L1 [25–27].

Despite the introduction of immunological treatment approaches aiming to stimulate anti-cancer immune reactivity and to overcome the immunological blockades imposed by the cancer, the multiplicity of mechanisms by which cancers can subvert these immunological treatment approaches continues to challenge immunotherapeutic efforts. This heterogeneity of tumor-induced immune suppressive mechanisms may warrant more than blockades of individual immune inhibitory routes to allow for effective immunological treatment for cancer. An alternative that is explored in this review is immunological treatment against precancerous lesions that are at high risk for secondary occurrences or progressing to cancer. It is not uncommon for multiple premalignant lesions to develop at various times due to the field effect of areas exposed to substances such as carcinogens [28]. These precancerous lesions are morphologically atypical and, while not yet malignant, are poised to progress to cancer. Unfortunately, the immunological status of the premalignant lesion environment is not well understood and similar observations in a number of instances have resulted in very differing interpretations. This review summarizes the immunological impact of premalignant lesions, both locally and systemically, the deficiencies in what is understood about the role of the immune infiltrate within premalignant tissues, and summarizes studies that have explored the feasibility of immunological treatments to prevent secondary occurrences of premalignant lesions and to prevent premalignant lesion progression to cancer. This could highlight a missed opportunity of preventing cancer by immunological treatment in presumably a less immune subversive environment than in the more challenging immune-hostile cancer environment.

Immunological milieu of premalignant lesions

Substantial efforts have been exerted on cancer prevention such as through lifestyle modifications to include improved diet, smoking cessation and reduced sun exposure. Less emphasis has been placed on immunological approaches to prevent cancer development or

progression prior to when cancers subvert immune defenses. An advancement toward this effort is the relatively recent availability of HPV vaccines, which aim to prevent cervical cancer, but also can become effective in preventing other HPV-associated malignancies such as squamous cell carcinomas (SCC) of the head and neck [29,30]. However, there remain non-HPV-associated malignancies that might also be preventable in individuals that are at high risk for development of cancer.

Premalignant lesions are tissues that are not yet malignant, but can progress to become malignant. Examples of these precancerous tissues include polyps in the colon, actinic keratosis of the skin, dysplasia of the cervix, metaplasia of the lung, and leukoplakias of the mouth. Premalignant lesions of the oral cavity, including leukoplakias and erythroplakias, are now routinely screened for during dental examinations [31]. Also routine are colonoscopies to detect colon polyps to, in turn, reduce colon cancer [32,33]. Dysplasia of the cervix is screened for by Pap smears [34]. While standard treatment for these premalignant tissues often includes their excision, such treatment does not remove premalignant cells that have not yet been detected and often does not prevent development of secondary lesions. Very few studies have examined the possibility of adapting immune therapeutic approaches for individuals with premalignant lesions that are at high risk of developing cancer. In fact, few studies have examined the immune environment of premalignant lesions or when, in the course of their progression to cancer, the immune inhibitory environment that is so prominent in cancer becomes established.

One study that compared the immunological microenvironment of intraepidermal carcinomas and SCC showed an increased content of T-cells, and in particular $CD8^+$ T-cells, within the lesions compared to the levels of these cells in cancer tissue [35]. In a separate study, premalignant oral leukoplakias were shown to be infiltrated by $CD3^+$ T-cells, with those containing lower numbers of $CD3^+$ cells having a higher incidence of progression to cancer [36]. It has also been shown that leukoplakias with dysplasia and oral SCC have a higher dendritic Langerhans cell and T-cell content than leukoplakias without dysplasia [37]. The conclusions of such studies suggest that the higher level of immune cell infiltration is indicative of ongoing immune reactivity against premalignant lesions and against cancers. However, additional studies are needed to determine whether this immune reactivity is a beneficial response that aims to protect against tumor development or whether the response could promote tumor development. Supportive of conclusions that immune cell presence within lesions could be an attempt to limit lesion progression are results of studies showing premalignant oral lesion tissues of patients and of a mouse model of premalignant oral lesions that progress to cancer contained increased levels of Th1 and inflammatory cytokines compared to levels within oral cancers [38].

Studies have, however, shown pro-tumorigenic effects of the immune response in precancerous lesions. Such studies have often focused on precancerous states of the gastrointestinal tract. Barrett's esophagus is a premalignant condition that is considered to arise from chronic inflammation and carries a high risk of progression to esophageal adenocarcinoma. Studies of the immune phenotypes in this progression have shown Barrett's esophageal tissues contain an elevated pro-tumorigenic Th2 immune phenotype, but this shifts once cancer has developed to a less activated T-cell phenotype that consists of

a mixed Th1 and Th2 cytokine profile [39]. In addition, infiltration by M2 macrophages and Treg cells was suggested to contribute to esophageal cancer development in a rat model of chronic duodenal content reflux esophagitis [40]. Similarly, studies with *Helicobacter pyroli*-infected patients having precancerous gastric lesions and H. *pyroli*-infected mice concluded that increased myeloid cell infiltration and increased IFN-γ expression could be contributing to progression of lesions toward a more cancerous state [41]. This progression of lesions toward cancer in spite of an increase in IFN-γ is paradoxical since IFN-γ is typically considered to be important in the defense against cancer. Gene expression profiles of colon polyp tissues and unaffected colon mucosa of patients having colon polyps showed significant overlap of changes in gene expression compared to gene expression profiles of healthy patients [42]. A large proportion of these alterations in gene expression were associated with immune inflammatory responses, leading the authors to suggest that the pro-inflammatory expression can promote the development of additional polyps in the unaffected colon mucosa of patients with polyps. However, in contrast with this suggestion of inflammation-promoted development of polyps, patients with ulcerative colitis were shown to have a similar frequency of developing polyps as did healthy controls, although, the histological types of polyps differed with an increase in inflammatory (pseudo) polyps [43]. This study also showed no increase in the incidence of adenomas among Crohn's colitis patients.

Studies indicating immune involvement in progression of premalignant states toward cancer have also been conducted in nongastrointestinal tract sites. Using the TRAMP mouse model that, upon puberty, progressively develops hyperplasia, prostatic intraepithelial neoplasia and carcinoma, the presence of T-cells was shown to facilitate that the progression process [44]. The requirement of T-cells for this progression was demonstrated through multiple means, including using T-cell-deficient crosses or T-cell receptor-deficient crosses of the TRAMP mice, and with T-cell reconstitution studies of these immune deficient mice. Studies with a different murine model of prostatic hyperplasia similarly suggested immune involvement in stimulating prostatic epithelial proliferation, but in this model, the inflammatory reaction was mediated by macrophage-derived IL-1 [45]. Macrophage recruitment was also suggested to promote the formation and progression of pancreatic premalignant lesions [46].

As described above, substantial information is now available about the immune content within precancerous tissues. Overall, inflammation along the gastrointestinal tract appears to have a closer connection to progression of premalignant states to cancer than what has been described for other sites. Such inflammation-associated disorders with increased risk of cancer include Barrett's esophagus, Crohn's disease and ulcerative colitis [47,48]. While there appears to be a closer link between inflammation along the gastrointestinal track and cancer development, there have not been analyses of whether, in fact, there are site-specific differences in how the immune infiltrate contributes to progression of precancerous sites to cancer. In fact, a significant weakness in most of the above-described studies analyzing the immune milieu of premalignant tissues is that much of the information about the immune infiltrate is correlative and lacks the detailed phenotypic or functional analyses that are needed to delineate the possible contribution of the infiltrating cells to the cancerous progression of premalignant lesions. Consequently, there remains uncertainly as to the role

of the immune infiltrate in premalignant tissue and the consequences of the immune infiltrate in the progression to cancer versus in protection from cancer progression.

Systemic immune alterations associated with premalignant lesions

The increase in immune reactivity within precancerous lesions does not appear to be limited to the immediate lesion site. Levels of inflammatory indicators such a C-reactive protein and IL-6 were shown to be increased in the peripheral blood of subjects with Barrett's esophagus and these increases were associated with a higher risk of the premalignant state progressing to esophageal adenocarcinoma [48]. Subjects with premalignant oral lesions, which are relatively small-sized lesions, have increased levels of inflammatory mediators TNF-α and IL-6 in their saliva, although salivary levels of these cytokines were shown to be higher in subjects with oral SCC [49]. A separate study similarly showed increased levels of TNF-α in saliva of subjects with premalignant oral lesions and cancer, but also showed that TNF-α levels were increased in the serum of these subjects [50]. These studies are in agreement with other studies showing increased splenic and regional lymph node pro-inflammatory activity with a Th1 and Th17 phenotype in a carcinogen-induced premalignant oral lesion animal model and in the blood of subjects with premalignant oral lesions [51–54]. However, in these latter studies, the regional and systemic pro-inflammatory milieu that is characteristic for mice and patients with premalignant lesions subsides upon development of oral cancer. Studies to assess the mechanism by which premalignant oral lesion cells alter cytokine levels systemically demonstrated that the stimulation of Th1 and Th17 cell-associated cytokines was through soluble mediators produced by premalignant lesion cells [52,55]. The induction of some, but not all, of the inflammatory mediators was blocked by inhibiting cyclooxygenase in premalignant lesion cells, suggesting that lesion cell-derived PGE_2 could be contributing to some of the systemic inflammation [56].

Impact of immune cells in premalignant lesions on progression to cancer

Whether or not the immune infiltrates within premalignant lesions are activated to react against the lesions versus being inhibitory to anti-lesion reactivity, as is seen in cancers, is not established. Oral leukoplakias were shown to not only have an increase in tumor-associated macrophages and T-cells compared to healthy normal biopsies, but these cells exhibited a M1 and Th1 phenotype, suggesting a beneficial anti-tumor potential that is driven by the Th1 phenotype [57]. In contrast, a mouse model of premalignant lung adenomas showed infiltration by immature macrophage-lineage cells that support the premalignant cells and produce inhibitory mediators such as TGF-β [58]. Likewise, an immune inhibitory state was demonstrated within HPV-associated respiratory papillomas in patients, as demonstrated by increased levels of Treg with suppressive activity and an increased Th2-like milieu [59]. This study also showed increased expression of the PD-1/PD-L1 inhibitory axis and indications of immune exhaustion. An assessment of premalignant pancreatic cysts having the potential to progress to cancer demonstrated infiltration by $CD4^+$ and $CD8^+$ cells, and was interpreted to indicate immune reactivity within the lesions [60]. However, this study also found increased levels of circulating MDSC and Treg cells, and indication of T-cell exhaustion in some of the patients. The culmination of the above studies suggest that there may be immune reactivity generated against

premalignant lesions, but the transition to immune inhibitory mechanisms could be occurring prior to the progression to cancer.

The role of Th17 cells in either promotion or defense against premalignant lesions and their progression to cancer is unsettled. An increased Th17 phenotype has been demonstrated for patients with premalignant oral lesions and in animal models with oral lesions, with these levels subsiding in the oral cancer environment [38,54,55]. Treatment of mice bearing carcinogen-induced oral lesions with IL-23 and a TGF-β receptor antagonist to sustain the Th17 milieu increased not only levels of Th17 cells, but also IFN-γ-expressing $CD8^+$ cells [51]. Furthermore, this treatment to sustain a Th17 phenotype reduced lesion progression to cancer, suggesting a protective role of Th17 cells. Ironically, shifting TGF-β in the opposite direction toward increased TGF-β expression in premalignant squamous papillomas similarly induced Th17 cells and IFN-γ-expressing $CD8^+$ cells, and induced regression of the papillomas [61]. In contrast, a different study in which TGF-β signaling was interrupted by deletion of Smad4 in T-cells showed increased levels of inflammatory and inhibitory cytokines, increased levels of Th17 cells and spontaneous development of premalignant lesions with the gastroduodenal regions of mice [62]. This study concluded that the Th17 activity could be associated with the development of the premalignant lesions.

Clearly the functional contribution of the immune infiltrate within lesions has yet to be fully defined as there are studies concluding protective roles of the infiltrate and there are studies concluding that the infiltrate promotes lesion progression to cancer. Most of the above-described studies have relied on correlative associations between what has been analyzed about the immune infiltrate of premalignant sites and likely functions that the immune phenotype may possess. Therefore, it is important to conduct more detailed phenotypic analyses that can be used to better indicate possible functions of the lesion-infiltrating immune cells. Unfortunately, many correlative studies between the immune infiltrate and their role in cancer progression lack sufficient phenotypic and functional analyses of the immune infiltrate to provide insight into whether the immune infiltrate under study would have a likelihood of protecting against progression to cancer, or whether it would have a likelihood of supporting cancer development. Thus, caution must be used in making conclusions based on correlative analyses as to the role of the immune infiltrate within premalignant lesion sites. Despite this uncertainty, it appears that the premalignant lesion immunological environment is less hostile to the feasibility of skewing the immune state to be protective than is the cancer environment and, thus, immunotherapeutic approaches to prevent secondary premalignant lesions or lesion progression to cancer should be more fully explored as immunological treatments for cancer.

Immunological treatment approaches for premalignant lesions

While studies have documented immune alterations, including increased immune activity, in premalignant lesions, studies to determine the feasibility of immunotherapeutic approaches to treat lesions, or to prevent their re-occurrence or progression to cancer have been few. The feasibility of immunotherapeutic approaches by which to treat premalignant lesions and to prevent progression to cancer is supported by several studies showing the sharing of tumor antigens between premalignant lesions and their cancer counterparts. For example,

squamous dysplasias have been shown to express some of the same tumor antigens as digestive tract carcinomas, especially esophageal squamous cell carcinoma [63]. Similar tumor antigens were expression by premalignant oral lesion of patients as are seen on head and neck squamous cell carcinomas [64]. Likewise, there was sharing of tumor antigens between premalignant oral lesions of a carcinogen-induced tongue lesion mouse model and the tongue cancers that developed from these lesions [65].

The few studies that have considered immunotherapy for treatment of premalignant lesions and to prevent their progression to cancer have had varied results. Topical application of the agents, Imiquimod and Diclofenac, stimulates cytokine production and can trigger regression of premalignant skin actinic keratosis lesions [66,67]. Studies have also suggested that the increase in $CD8^+$ cells within intraepidermal carcinomas, which are an earlier stage of cancer, might be responsible for their regression following dermal cytokine-inducing treatments [35]. An indirect form of immunotherapy for mice bearing premalignant oral lesions has aimed to temper the inflammatory state by inhibiting production of prostaglandins, which can function both as inflammatory mediators and as inhibitors of Th1 immune reactivity [56]. This approach stimulated levels of IFN-γ-expressing $CD8^+$ cells locally within regional lymph nodes and distally in the spleen. This coincided with a delayed progression of premalignant lesions toward cancer. Consistent with these studies was demonstration that administration of selective inhibitors of cyclooxygenase-2 (COX-2) to rats diminishes the carcinogen-induced inflammatory NF-kB signaling pathways and retards development of colonic tumors [68]. Contrasting with these latter two studies, administration of the select COX-2 inhibitor celecoxib to a mouse model of *Helicobacter-associated* precancerous lesions tempered the immune inhibitory effects of PGE_2 on expression of the Th1 cytokine IFN-γ and, in turn, accelerated development of the precancerous lesions [69]. When, instead, mice were treated with synthetic analogs of PGE_2, levels of lymphocytic infiltration, inflammation, and production of IFN-γ decreased as did gastric premalignant immunopathology. In a population-based, case-controlled study on the effectiveness of non-steroidal anti-inflammatory drugs in subjects with Barrett's esophagus, whose progression to esophageal adenocarcinoma has been strongly shown to be inflammation-associated, there was shown to be no protective effects of the anti-inflammatory treatment on the incidence of cancer development [70]. In fact, treatment with anti-inflammatory compounds was also found not to diminish the development of Barrett's esophagus in subjects with gastroesophageal reflux disease, an inflammatory state that is the precursor to Barrett's esophagus [71]. However, recognizing that there have been inconsistencies in some results of analyses of the effectiveness of non-steroidal anti-inflammatory compounds and aspirin on the development of cancer in subjects with Barrett's esophagus, a meta-analysis was conduction of 9 separate studies [72]. This analysis showed a beneficial cancer preventative effect of using cyclooxygenase inhibitors and indicated the need for better randomized, controlled studies to obtain more definitive results of the effectiveness of interfering in inflammation to prevent esophageal cancer in Barrett's subjects. Overall, studies to intervene in inflammatory responses leave unresolved beneficial or detrimental possibilities of treatment approaches that target inflammation to alter the progression of premalignant states toward cancer, despite the clear demonstrations of the presence of immune activation.

Other than the HPV vaccines currently used to prevent HPV infection and associated cancers in human subjects, there are no approved vaccines for those who are already infected with HPV or which already have HPV-associated premalignant or malignant disease. A vaccination study in which patients with low-grade premalignant cervical abnormalities were vaccinated with a HPV16-synthetic long-peptide vaccine representing the E6 and E7 oncoprotein sequences showed HPV16-specific IFN-γ T-cell responses [73]. After re-vaccination at 1 year, Th1 responses remained, although enthusiasm for the re-vaccination was dampened by the Th2 cytokine response that also developed upon revaccination. In a study using a mouse HPV tumor model to assess both immunological and clinical responses, peptide mixtures of the HPV E7 oncogene were shown to stimulate both antibody and cellular immune responses reactive to HPV constructs and to limit tumor development [74]. While this latter study demonstrated the feasibility of HPV vaccine approaches to limit tumor development, it did not address the impact of vaccination on eliciting reactivity to premalignant lesions and their progression to cancer.

Only a few studies have tested non-HPV vaccine strategies to prevent premalignant lesion progression to cancer. Using a carcinogen-induced premalignant oral lesion mouse model, administration of a premalignant lesion-pulsed dendritic cell vaccine increased Th1 and Th17 immune reactivities and slow progression to cancer [75]. However, dendritic cells that were pulsed with normal tongue epithelium lysate also induced this immune activation, although the premalignant lesion-pulsed dendritic cells induced a delayed, yet substantial stimulation. Possible interpretations for these results could be that the delay in the response to lesion-pulsed dendritic cell vaccination could reflect an increased resistance to immune exhaustion. A TgMMTV-neu mouse model that develops spontaneous mammary cancers expressing neu, insulin-like growth factor (IGF) binding protein 2 and IGF receptor-I was used to test a vaccine consisting of peptides derived from these proteins [76]. Administration of the peptide vaccines to mice with lesions that were not yet palpable, but which were destined to develop palpable breast cancer, showed the highest effectiveness by the multipeptide vaccine than individual peptide vaccines at preventing development of palpable lesions. This protection was $CD4^+$ cell-mediated, with the vaccinated mice demonstrating antigen-specific IFN-γ responses. Overall, such immune modulatory and vaccine strategy studies support the feasibility of using immunological means to prevent the emergence of secondary lesions that are not yet macroscopically detectable and to prevent progression of premalignant lesions to cancer. However, these studies also highlight the paucity of studies focused on early immune intervention in individuals with premalignant lesions that are at high risk of developing secondary lesions or cancer.

Conclusions

The cancer milieu has been well studied, with multiple documentations of immune infiltration into the tumor, but also showing tumor evasion and subversion of these immune defenses. In such an immune suppressive environment of the tumor, mounting immune reactivity through immunotherapeutic approaches is challenging. There are, however, a number of premalignant lesion conditions that pose a high risk of developing into cancer. Unfortunately, compared to what is known about the immunology of cancers, far less is knows about immune reactivity to premalignant lesions. Despite the increased emphasis on

prevention of cancer, the testing of immunotherapies for the treatment of premalignant lesions so as to prevent the occurrence of subsequent secondary lesions or prevent their progression to cancer has been severely understudied. Thus, it is difficult to appreciate whether the premalignant lesion environment, which could be less immune subversive than the one for cancer, would be better suited for immunotherapeutic treatment approaches. As immunological treatment strategies are tested for cancer patients, the lessons learned from both successes and failures of these treatments, could potentially be applied to immunological treatments in a less hostile premalignant lesion state. The challenge will be in the design of clinical trials involving subjects with high risk premalignant lesions that can definitively determine the efficacy of immunological treatments at preventing secondary lesions and preventing progression of precancerous lesions to cancer.

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