Medical Hypotheses xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy

Transmission of "split anergy" from tumor infiltrating to peripheral NK cells in a manner similar to "infectious tolerance"

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ARTICLE INFO

Article history:
Received 10 March 2013
Accepted 19 May 2013
Available online xxxx

ABSTRACT

According to a new paradigm of carcinogenesis, a tumor arises not from transformed cell, but only from tumor initiating cells called cancer stem cells (CSCs), which can originate from tissue stem cells. CSC are resistant to conventional therapy and after treatment form new tumors and give rise to metastases. Only natural killer (NK) cells are capable of lysing CSCs, but within different tumor types these cells experience a condition known as "split anergy", whereby the NK cells lose the ability to kill CSCs and being to produce cytokines. As a result, uncontrolled tumor growth arises and tumor stroma accumulates anergic NK cells. We hypothesize that anergic tumor infiltrating NK (TINK) cells transmit their property to naïve NK cells by infecting" them with a state of "split anergy" in a similar manner as T conventional cells are transformed into T regulatory cells during the process of "infectious tolerance". Anergic TINK cells egress from the tumor stroma via the lymphatic system, where they reach regional lymph nodes and transmit their properties to naïve NK cells, which in turn become anergic toward CSCs and lose immunosurveillance functions. The mechanisms proposed for this hypothesis and the methodological approaches for confirming the idea are presented in this issue.

Background

The discovery of a minor cell population in tumors with features of tissue stem cells, which are called cancer stem cells (CSCs), has led to a new paradigm of carcinogenesis. According to this notion, many cancers (if not all) arise from CSCs, which renew themselves by asymmetric division (hierarchical model of carcinogenesis) [1–5]. A hierarchical model is distinct from a stochastic model, as the former is based on tumors arising not just from any tumor cell, but rather only from CSCs. The CSCs have features common with tissue stem cells (TSCs), including the ability to self-renew and differentiate, activate telomerase expression, activate antiapoptotic mechanisms, enhance activity of membrane transporters, and migrate (metastasize). The mechanism responsible for the transformation of TSCs into CSCs remains elusive; however, together with mutagen input, several other factors are suspected in the initiation of this process, namely, reactive oxygen and nitrogen species, prostaglandin E2, and some cytokines and chemokines produced by proinflammatory cells, which infiltrate into the site of chronic inflammation [6–9].

Recently, CSCs have been found in various malignancies, including gastric cancer [10], colorectal cancer [11], hepatocarcinoma [12], pancreas cancer [13], prostate adenocarcinoma [14,15], brain tumor [16], head and neck cancer [17], chronic and acute leukemia [18,19], lung cancer [20,21], and breast cancer [22–25]. CSCs are resistant to the chemo- and radiotherapy [26], and after a course of conventional treatment, CSCs remain intact and form new tumors that can give rise to metastatic lesions [27–29].

The search of drugs that specifically eliminate CSCs presents an urgent task of contemporary pharmacology [30–33]. It is generally believed that the activation of the immunosurveillance system through natural killer (NK) cells, which represent the major effectors at the intersection of innate immunity, represents a principal solution to this problem. According to recent reports, NK cells that are activated by IL-2 or IL-15 become capable of lysing CSCs present in glioblastoma [34] and melanoma [35], characterized by the absence or weak expression of HLA class I. Moreover, normal NK cells have been shown to lyse embryonic stem cells (ESCs; mouse model) [36] as well as human mesenchymal stem cells (MSCs) and dental pulp stem cells (DPSCs). They can also lyse pluripotent stem cells [37], but not their differentiated progeny. The cytolytic activity of circulating or tumor infiltrating NK (TINK) cells, as measured by the *in vitro* lysis of the erythroleukemia cell line K562 or autologous tumor cells in different types of tumors, is considerably low [38–44].

Studies by Jewett et al. [37,45–48] have shown that NK cells lose the ability to lyse NK sensitive targets, including K562 cells,

Abbreviations: (B)CSC, (breast) cancer stem cells; DPSC, dental pulp stem cells; ESC, embryonic stem cells; ISA, infectious split anergy; LEC, lymphatic endothelial cells; MSC, mesenchymal stem cells; TINK, tumor-infiltrating NK cells; TSC, tissue stem cells.

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0306-9877/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.mehy.2013.11.019

oral squamous carcinoma stem cells, MSCs, DPSCs, ESCs, and induced pluripotent stem cells after direct contact with them. A decrease in the cytolytic activity and IL-6 secretion as well as production of IFNγ can change the phenotype from $CD16^{+}CD56^{+/dim}CD69^{-}$ into $CD16^{-}CD56^{dim/-}CD69^{+}$, which phenomenon was named "split anergy". Split anergy can also be been induced by the simultaneous administration of IL-2 and anti-CD16 antibodies or monocytes on freshly isolated NK cells.

Studies suggest that NK cells, despite their well-recognized function as the first line of defense as effectors against viral infection and malignancy, operate as managers of the regenerative process in injured tissue by destroying altered tissue stem cells that are incapable of differentiating or by regulating their quantitative excess with concomitant support of SC differentiation and promotion of tissue regeneration after gaining cytokine producing function. Any alteration of this activity (e.g., split anergy) will lead to the development of chronic inflammation and dampening of local regeneration, which can cause continuous tissue damage and recruitment of immune effectors. Moreover, chronic inflammation by itself is considered as a necessary condition for carcinogenesis through the stimulation of TSCs transformation into CSCs [49].

Thus, NK cells view CSCs as altered TSCs, eliminate them after the first contact, and then acquire a state of split anergy. The condition of split anergy can also be induced by an immunosuppressive tumor microenvironment formed by the presence of tumor-associated fibroblasts [50], suppressive endothelial cells [51], tumor-associated macrophages [52,53], tolerogenic dendritic cells [54], FoxP3+ Treg-cells [55,56], and myeloid-derived suppressor cells (MDSCs) [57]. As a result, uncontrolled tumor growth arises and tumor stroma accumulates anergic NK cells. Comparing this condition to the decreased cytolytic activity of circulating NK cells, it is likely that the conditions of NK split anergy arise from tumor tissue into the periphery through a specific mechanism. We hypothesize that anergic TINKs transmit their characteristic properties to newcomer naïve NK cells by "infecting" them with a state of split anergy in a similar manner as T conventional (Tconv) cells are transformed into T regulatory (Treg) cells, in a process called "infectious tolerance". Infectious tolerance represents a phenomenon where natural Tregs bestow Tconv cells with suppressive features, which consequently allows for the propagation of suppressive abilities among Tconv cells [58].

Hypothesis

TINKs are in a state of split anergy, which is described as a condition where the cells lose their ability to kill CSCs and subsequently acquire cytokine production capabilities [48]. These conditions lead to unrestricted CSC maintenance and consequently tumor growth. Conventional anti-tumor therapy (chemotherapy, surgery, or radiotherapy) do not prevent possible relapses in the form of metastatic disease, which involves CSC migration and spreading. We propose that the split anergy of NK cells is disseminated from TINKs to their peripheral counterparts. This process occurs in a similar way to "infectious tolerance" of Treg cells [58]. We hypothesize that anergic TINKs egress from tumor stroma through the lymphatic system, reach regional lymph nodes, and transmit their properties to naïve NK cells, which in turn become anergic toward CSCs and do not have proper immunosurveillance functions. For convenience, in the given circumstances, we will refer to this term as an "infectious split anergy" (ISA).

Questions:

1. Do TINKs egress from the tumor to the peripheral blood circulation? We hypothesize that the migration of TINKs is a prerequisite for cell–cell contact dependent interactions with peripheral NK cells.
2. What is the mechanistic basis of infection tolerance transmission between TINKs and peripheral NK cells?
3. What is the role of this process in the relapse phenomenon?
4. Can NK cells already "infected" transmit this effect to secondary and further "victims" and to what extent? Can "victims" in turn perform the same effect? Does it weaken over time?

The first part of the proposed hypothesis and the approach used to explore it will help to elucidate whether TINKs are fully resident or can migrate to some extent out of the tumor. The second part of the investigation is aimed at investigating the role of such NK cells in acquiring "split anergy" in the tumor microenvironment and their ability to propagate ISA.

Mouse model of TINK egression from tumors

1. Isolate NK cells from syngenic mice and label them with a stable marker (e.g., a marker suitable for further flow sorting; alternatively, a marker applicable to magnetic cell separation with cell capture systems such as CherryPicker®).
2. Inject them together with an appropriate tumor cell line suspension. In our work, we would like to concentrate on breast cancer (see below), but for this purpose the general plan of investigation is described. Notably, existing NK cells in recipient mice should first be depleted with an appropriate antibody (e.g., anti-NK1.1 or anti-asialo-GM1 antibodies [59,60]), in order to saturate the tumor with the maximum amount of labeled NK cells. When the tumor achieves sufficient size, it should be resected and transplanted into the second recipient (the whole tumor).
3. If NK cells have the ability to migrate out of the tumor, then labeled NK cells can be observed in the secondary lymphoid organs.

These experimental conditions will be closer to the conditions that can occur *in vivo*. Experiments on mice, as described above, is a prelude for further investigation that is aimed to dissect the role of CSCs in the acquisition of split anergy by TINKs. The results we obtain can presumably be extrapolated to other cancer types; however, the specific features and characteristics of each tumor type will need to be considered.

TINKs do not preserve cytolytic function and have an altered phenotype compared to peripheral NK cells. Previous study assessing these cells in non-small lung cancer found that NKp30, NKp80, CD16, NKG2D, and DNAM-1 were down-regulated on TINKs compared to peripheral blood-derived NK cells from cancer patients or healthy donors, respectively. The cytolytic activity against K562 or autologous tumor cells was also reduced [61]. Moreover, a previous microarray study has detailed the differences between TINKs and peripheral NK cells [62].

Breast cancer is a widely distributed malignancy, and the role of CSCs in breast cancer has been thoroughly investigated. The breast cancer stem cell (BCSC) phenotype is well established [63] and thus methods have already been developed for investigation, which partially intersect with methods used to study CSCs in other malignancies [64].

In vitro investigation of TINK and NK cell, preincubated with BCSC, influence on the peripheral NK cells

The second part of the research is aimed to study the processes on isolated human NKs and BCSCs *in vitro*.

1. Isolate BCSCs from tumor tissue and confirm their identity using method previously reported [65]. Autologous TINKs and peripheral NK cells need to be thoroughly investigated to

Please cite this article in press as: Belyaev NN, Abramova VA. Transmission of "split anergy" from tumor infiltrating to peripheral NK cells in a manner similar to "infectious tolerance". Med Hypotheses (2013), http://dx.doi.org/10.1016/j.mehy.2013.11.019