Calcineurin Signaling as a Negative Determinant of Keratinocyte Cancer Stem Cell Potential and Carcinogenesis

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Abstract
Calcineurin is the only known serine-threonine phosphatase under calcium–calmodulin control and key regulator of the immune system. Treatment of patients with calcineurin-inhibitory drugs like cyclosporin A and FK506 to prevent graft rejection dramatically increases the risk of cutaneous squamous cell carcinoma, which is a major cause of death after organ transplants. Recent evidence indicates that suppression of calcineurin signaling, together with its impact on the immune system, exerts direct tumor-promoting effects in keratinocytes, enhancing cancer stem cell potential. The underlying mechanism involves interruption of a double negative regulatory axis, whereby calcineurin and nuclear factors of activated T-cell signaling inhibits expression of ATF3, a negative regulator of p53. The resulting suppression of keratinocyte cancer cell senescence is of likely clinical significance for the many patients under treatment with calcineurin inhibitors and may be of relevance for other cancer types in which altered calcium–calcineurin signaling plays a role.

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Introduction
Squamous cell carcinomas (SCC) have a very high incidence in the human population, encompassing skin, oral and esophageal cancer, squamous (bronchial) lung carcinoma, and carcinoma of the cervix and other parts of the urogenital system. A distinguishing feature of these tumors is their high level of heterogeneity, with self-renewing cell populations admixed with cells at different stages of differentiation. The aggressiveness of these tumors, and their resilience to therapy, are likely determined by a dynamic equilibrium between stem cell populations and their daughter cells (1–3). In the skin, as in other tissues, cells harboring oncogenic mutations can remain quiescent for long periods of time (4).

Important mechanisms involved in restraining cells with oncogenic potential are the induction of cellular senescence (5) and/or differentiation (6). Counteracting these failsafe mechanisms are conditions of perturbed tissue homeostasis, such as those resulting from wound healing (7) or inflammation (8). The immune system is also thought to play a major role in preventing or limiting tumor spread (9).

Calcineurin–Nuclear Factors of Activated T-cell Signaling and Biological Function
Calcineurin is the only known serine-threonine phosphatase under calcium–calmodulin control (10). It is a heterodimeric enzyme formed by a catalytic [calcineurin A (CnA)] and a Ca²⁺-binding regulatory subunit [calcineurin B (CnB); ref. 11]. CnA shows a highly conserved multidomain structure: The catalytic domain (residues 70 to 328) is followed by the CnB-binding domain (residues 333 to 390) and a C-terminus regulatory domain (residues 390 to 521). This regulatory domain, in turn, can be divided into 2 subdomains: a calmodulin-binding and an autoinhibitory subdomain (11). Three isoforms of CnA exist, α (neural), β (ubiquitous), and γ (testis specific), which share more than 80% of identity in the catalytic and regulatory regions (11). CnB is expressed in 2 isoforms, CnB1 (ubiquitous) and CnB2 (testis specific). CnB is absolutely required for calcineurin enzymatic activity (11), even if its role in calcium-dependent activation remains unclear.

Among the proteins that are dephosphorylated as a consequence of calcineurin activation are the nuclear factors of activated T cells (NFAT). Increased calcineurin activity promotes the localization of NFATs to the nucleus, which is counteracted by the phosphorylation of these factors by a number of kinases such as GSK3, CK1, p38, JNK1, and DYRK1A (10, 12–14). This opposite mode of regulation may explain why induction of NFAT-dependent transcription by calcineurin activation is not immediately associated with increases in intracellular calcium levels but requires a more prolonged period of time (10).

Studies on the biological function of calcineurin have been greatly facilitated by the use of the inhibitory drugs...
cyclosporin A (CsA) and FK506 (10). Several endogenous calcineurin inhibitors have also been reported. Among these is calcipressin (CALP1), also known as the DSCR1 gene product, located in the Down syndrome candidate region of human chromosome 21 (15). This protein binds directly to the calcineurin catalytic subunit and inhibits its activity (15). Importantly, calcipressin gene expression is under direct positive control of calcineurin–NFAT activity, so that this protein can function as part of a negative feedback mechanism for modulation of calcineurin signaling (15).

The function of calcineurin has been elucidated in great detail in T cells, but has also been studied in the hematopoietic, neuronal, myogenic, and vascular systems (10). Calcineurin activity has also been implicated in promoting keratinocyte differentiation (16) and, in vivo, in control of the hair cycle in concert with Notch signaling (17). Downstream of calcineurin, a specific NFAT isoform, NFATc1, has been functionally linked to maintenance of quiescent stem cell populations in the mouse hair follicle (18).

**Calcineurin–NFAT Involvement in Tumor Development**

A number of studies have shown that deregulation of calcineurin–NFAT signaling can play an important role in tumorigenesis. This pathway has been implicated in the control of tumor-associated angiogenesis. In mice with loss of calcipressin function, enhanced calcineurin activity leads to apoptosis of endothelial cells, with suppression of tumorigenesis (19). Treatment of these mice with CsA rescues the endothelial defect, restoring tumor growth (19). However, increased expression of calcipressin in mice with one additional copy of the gene has also been linked to tumor suppression through negative modulation of the vasculature (20). This has been proposed as a mechanism for the reduced susceptibility of Down syndrome patients to cancer development (20).

Specific NFAT isoforms have also been implicated in a number of human solid tumors or hematologic malignancies, through a variety of mechanisms that enhance the intrinsic tumorigenic properties of cells and/or alter their surrounding environment (21).

In the clinic, treatment of patients with calcineurin inhibitors to prevent graft rejection results in a dramatic increase of cutaneous SCC formation (22). In fact, cutaneous SCC is one of the most deleterious consequences of treatment with these drugs, historically accounting in some cohorts for up to 50% of deaths after year 4 from transplants (23). Interestingly, the combined use of CsA and psoralen-UVA in patients with psoriasis also causes a very high rate of skin SCC, which prompted discontinuation of this modality of treatment (24). The tumor-promoting effects of calcineurin-inhibitory drugs have been generally attributed to inhibition of the immune system and, in particular, T-cell function (see, for instance, refs. 25, 26). However, although the risk of cutaneous SCC in CsA-treated patients is 65- to 100-fold higher than in the normal population, the incidence of other skin tumors, like basal cell carcinoma (BCC) or melanoma, or that of internal malignancies, increases to a significantly lesser extent (22). Additionally, other more recently developed immunosuppressive drugs that do not affect calcineurin activity, such as mTOR inhibitors, have a much smaller impact on skin SCC formation (22). Thus, although important, immune suppression, per se, is unlikely to account for the selectively increased risk of cutaneous SCCs associated with calcineurin inhibition.

**Intrinsic Tumor-Suppressive Function of Calcineurin–NFAT in Keratinocytes**

In recent work, we have tested the possibility that, together with its function in keratinocyte growth and/or differentiation control (16–18), the calcineurin–NFAT pathway plays an intrinsic role in skin SCC tumor suppression. In support of this possibility, we found that mice with keratinocyte-specific deletion of the calcineurin B1 gene (CnB1), essential for calcineurin activity (17), have increased susceptibility to chemically induced carcinogenesis, with decreased latency, higher incidence and size of tumors, and earlier malignant conversion (27). In a xenograft model of SCC formation in severely immunocompromised mice, genetic and pharmacologic suppression of calcineurin–NFAT function was also sufficient to enhance tumorigenicity of primary human keratinocytes (HKC) expressing oncogenic H-rasV12 or cutaneous SCC cells (27).

The size of cancer stem cell populations is a likely determinant of the susceptibility to skin cancer development (28). Like primary keratinocytes, established cancer cell lines, including SCC cells, contain distinct subpopulations with different self-renewal capability (29, 30). As an in vivo assay of tumorigenic potential, H-rasV12-expressing HKCs, sorted for stem cell–associated markers, were injected in serial dilutions at the dermal–epidermal junction of severely immunocompromised mice. In control mice at 1 month after injection, cells formed only a few keratinized cysts with limited cellularity. By contrast, in the CsA-treated mice, the same cells gave rise to a striking number of “proliferative centers,” composed of keratinocytes with elevated Ki67 positivity. Cells isolated from these centers readily produced secondary tumors upon transfer into similarly immune-suppressed and CsA-treated mice. Similar results were obtained with sorted SCC cell lines (27).

**Antagonistic Role of Calcineurin and ATF3 in p53-Dependent Cancer Cell Senescence**

Cancer cell senescence is a failsafe mechanism against tumor development that can suppress cancer stem cell potential. Underlying its tumor-promoting effects, calcineurin–NFAT inhibition was found to suppress cancer cell senescence together with downmodulation of p53 expression, a key mediator of oncogene-induced senescence (27). Downmodulation of p53 occurred at both the protein and mRNA level. Although the best-studied mechanisms of p53 regulation are post-transcriptional (31), a perhaps less appreciated, but important, form of p53 regulation is at the level of p53 gene...
transcription (1, 32). Previous work showed that p53 gene transcription is under negative control of the AP-1 complex, specifically c-Jun and c-Fos, in both HKCs and SCC cells (33). c-Jun and c-Fos levels were unaffected in these cells by calcineurin–NFAT inhibition. However, expression of ATF3, a member of the ATF-cyclic AMP response element-binding family of transcription factors that can heterodimerize with AP1 family members (34), was sharply upregulated (27). Increased ATF3 expression was previously connected with tumor-enhancing effects as calcineurin–NFAT inhibition. These findings were not limited to the experimental situation. In fact, decreased senescence and increased ATF3 expression were also observed in a large cohort of SCCs from CsA-treated versus untreated patients (27).

Possible Dual Consequences of Calcineurin Suppression and ATF3 Upregulation on Intrinsic Cell-Regulatory Mechanisms and Cytokine Production

In most cells, ATF3 expression is very low and strongly induced in response to a variety of stress-related signals (34). In keratinocytes, transcription of the ATF3 gene is under direct negative control of NFATc1 (27), but other mechanisms are likely to participate in its regulation, such as UV exposure and production of reactive oxygen species. Like other key transcription regulatory molecules, ATF3 can either promote or suppress tumor development, depending on cell type and context (37). In addition, ATF3 is involved in immunity and inflammation through control of cytokine expression (34). Intriguingly, p53, which we have implicated as a significant target of ATF3 in keratinocytes (27), also modulates a number of cytokines with a known or likely role in control of proliferative potential (38). Downstream of p53, upregulation of the Notch 1 gene plays a key role in restricting keratinocyte self-renewing populations and promoting differentiation (39, 40), while, at the same time, influencing the surrounding stromal environment (41). Thus, a model can be envisaged whereby calcineurin–NFAT signaling plays a dual tumor-suppressing function in keratinocytes. On the one hand, inhibition of this pathway, through elevation of ATF3 expression, can suppress p53-mediated cellular senescence and expand cancer stem populations (Fig. 1A and B). On the other hand, calcineurin inhibition, through this or other mechanisms, could lead to a more permissive tumor-promoting environment (Fig. 1C).

Future Perspectives

A number of important questions need to be addressed. First of all, there is no explanation for the strongly increased risk for patients under treatment with calcineurin inhibitors of keratinocyte-derived SCC in the skin, as opposed to internal organs like the oral cavity. A likely culprit is exposure to UV light and its possible interplay with calcineurin–NFAT signaling in keratinocytes, either directly or through cytokine-mediated inflammation (Fig. 1C). A second unresolved issue is the basis for the selectively increased risk of cutaneous SCCs versus BCCs in patients under treatment with calcineurin inhibitors.
inhibitors. This risk could be linked to different cells of origin of the 2 types of tumors and the different role that calcineurin-NFAT may play in these cells. Finally, an exciting possibility for future studies is that the concomitant pharmacologic upregulation of the p53 and Notch pathways, known to induce senescence and/or differentiation (1), may be of clinical significance in reducing the risk of cutaneous SCCs in patients under treatment with calcineurin inhibitors and related predisposing conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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