New Aspects of Cyclosporin A Mode of Action: from Gene Silencing to Gene Up-Regulation

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Abstract: Cyclosporin A (CSA) has transformed clinical transplantation, both in term of success and of quality-of-life of the patient. Studies aimed to unfold the site of CSA action have shown that this molecule binds to cytosolic proteins of the cyclophilin family. CSA:cyclophilin complexes have a high affinity for calcineurin, a key enzyme in T-cell activation. By blocking the calcineurin activity, CSA prevents the induction of genes encoding for cytokines and their receptors. Thus, humoral and cellular immune responses are abolished, this resulting in the successful graft acceptance. Disappointingly, CSA and the other molecules as FK506, sharing the capacity to inhibit calcineurin, should be administered for all patient life, as tolerance to alloantigens is not achieved by these molecules. The long term utilization of this class of immunosuppressors increases the incidence of different tumors. The finding that CSA does not interfere with various biochemical pathways has prompted different groups to analyze a possible effect of CSA on molecules that might be involved in different functions of the immune response and/or in tumorogenesis. A new picture of CSA mode of action is emerging in which the immunosuppressor prevents the transcription of a group of genes, concomitantly inducing the transcription of another set. Here, we review the data and discuss the consequences of these new findings in term of T-cell activation mechanisms.

INTRODUCTION

Originally identified by screening for antibiotics from microorganisms, cyclosporin A (CSA) was found to suppress the immune system by altering activation of the genes that encode immune factors. Following the discovery of its immunosuppressive potential in the late 70s [1, 2], a major effort was undertaken to understand how this molecule interferes with an immune response. Clinical utilization of CSA was initiated even before the mechanism of its mode of action was discovered [3, 4]. As it will be discovered later, the advantage of CSA over the other immunosuppressors used in the 70ths in organ transplantation is its specificity for the cells involved in the immune response, and more particularly T-cells.

Twenty years after its first utilization, hundred thousands organ transplants have been successfully performed and it can now be concluded that CSA (and molecules sharing its mode of action) have transformed transplantation not only in term of efficiency but also in quality-of-life measurements of the patients [5]. A major disenchantment was the discovery that CSA did not induce tolerance towards the graft antigens. As discontinuation of CSA therapy is associated with graft loss due to rejection, the patients should be treated with the immunosuppressor for all their life. Thus, the benefits of CSA utilization, *i.e.,* better graft survival, better quality of life, have to be weighted against the risk of nephrotoxicity, hypertension, symptomatic hyperuricaemia and hyperlipidaemia which are associated with prolonged CSA utilization. Identical complications arise with an other immunosuppressor, tacrolimus or FK506 that shares the same mechanism of action as CSA. It should be however stressed that toxicity is a general feature associated with immunosuppressors therapy and is not restricted to CSA and FK506. As early as 1980, the risk of an increased incidence of lymphomas in patient under CSA therapy was forecast [6, 7]. Further studies confirmed that CSA-treated patients had an increased incidence of lymphoproliferative disorders and skin cancers, a common and dreaded complication [8, 9].

In addition to its utilization in clinics, CSA opened the access to the analysis of the mechanisms connecting membrane to nuclear events and solved the riddle of the "missing link" [10] between the biochemical events taking place at the membrane and the program of gene expression. As pointed out by Nabel [11] there is a discordance between the effects of the immunosuppressors that prevents the transcription of genes encoding for growth and differentiation factors involved in the immune response and the phenotypes of mice invalidated for the IL-2 gene or the nuclear factor of activated T-cells (NFAT). Thus, the effect of CSA and FK506 on T-cell activation is more complex than previously thought. In addition, CSA and FK506 affect also a large variety of cells belonging to the non-lymphoid lineage. This review attempts to summarize the current state of knowledge regarding newly discovered properties of CSA on selected gene activation in a variety of cells, not exclusively in the cells involved in the immune response.

CSA AND IMMUNOSUPPRESSION: A STORY OF UBIQUITOUS RECEPTORS AND A SPECIFIC PHOSPHATASE

Activation of T-cells is initiated by a cascade of biochemical events resulting from TCR occupancy by its

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peptide/MHC ligand and is modulated by co-engagement of other T-cell surface molecules such as costimulatory and integrin receptors. These interactions lead to an ensemble of intracellular signals which integration results in nuclear transcriptional changes, cytoskeleton modification, cytokine production, proliferation and differentiation. One of the earliest TCR-mediated activation event is the triggering of protein tyrosine kinases (PTKs). Phosphorylation on tyrosine residues of adaptor proteins and enzymes results in the formation, at the membrane, of a multimolecular complex nucleated by the protein linker for activation of T cells (LAT). Two main pathways are then activated. One resulting in signaling through PLC/Ca^{2+}/calcineurin [12, 13], the second proceeding in MAPKs activation through Ras and Rac small GTPases (reviewed in [14, 15]).

The principal immunological target of CSA is the T-cell subpopulation. Early studies revealed that CSA blocks the transcription of the interleukin-2 gene [16, 17, 18, 19]. It was rapidly realized that CSA interferes with a Ca^{2+}-dependent pathway downstream of diacylglycerol metabolism and linked to IL-2 gene expression (reviewed in [20]). The transcription of this gene depends on the binding of different proteins to specific DNA sequences located in its promoter region. The heterologous transcription factors NFAT and AP-1 coordinately regulate IL-2 gene expression throughout cooperative binding to juxtaposed DNA recognition elements. The 5'-distal NFAT site is the prime regulatory element for IL-2 gene maximal expression. To this site binds a complex formed by NFAT and AP-1, an heterodimer of different members of the jun and fos families [21]. A phosphorylated NFAT form pre-exists in the cytoplasm of resting cells. Upon T-cell triggering and calcineurin activation, NFAT is dephosphorylated [22, 23, 24] and translocates to the nucleus where it combines with AP-1 [21]. AP-1 is absent in resting T-cells and is rapidly induced following TCR triggering. Its synthesis depends on the activation of Ras/Rac pathways that stimulate several MAP kinases. CSA and FK506 do not block the Ras pathway and AP-1 activation ([21, 22, 24, 25], reviewed in [13]). Strikingly, CSA prevents the rapid disappearance of AP-1 from the nucleus [26].

The molecular target of CSA is a protein belonging to the cyclophilin family [27, 28, 29]. These highly conserved and abundant proteins bind CSA with high affinity and form a stable complex that, in turn, associates with calcineurin, a calmodulin-Ca^{2+}-dependent phosphatase [30, 31]. A

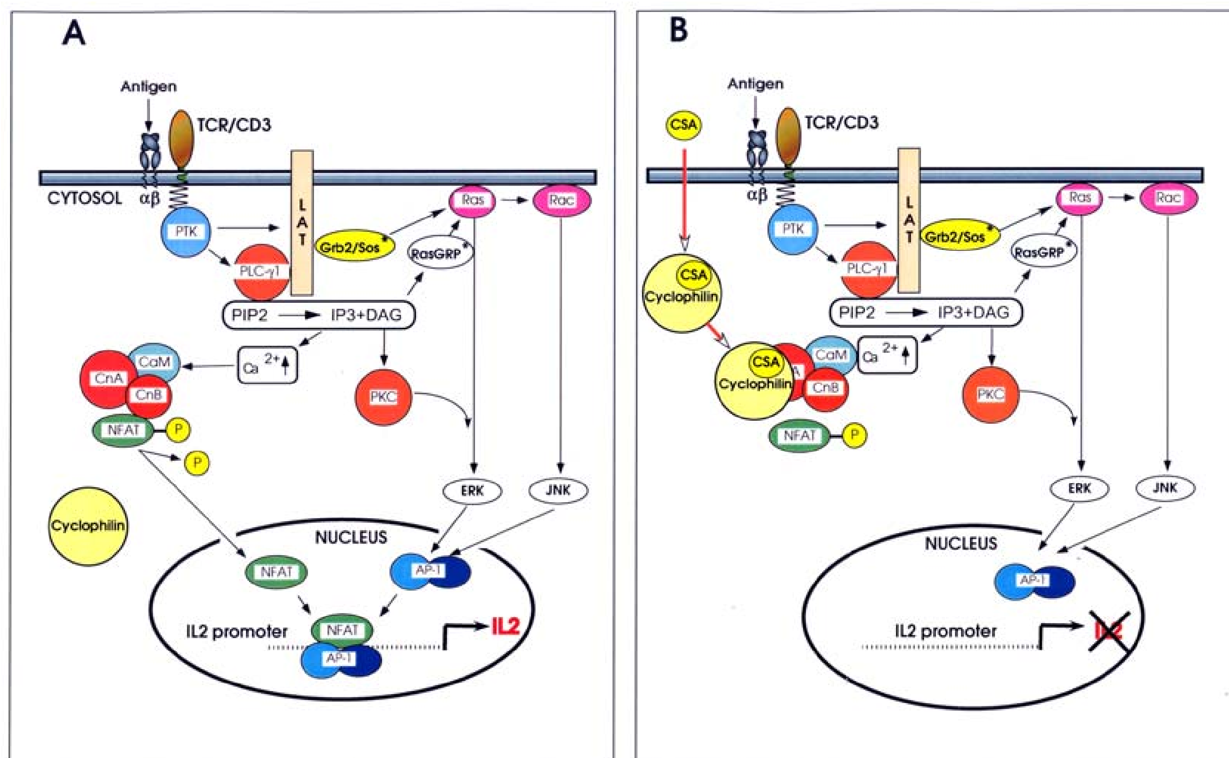

Fig. (1). Mechanism of T-cell activation and the site of action of CSA and FK506

A: TCR accupancy leads to PTKs activation and to the increase in cytosolic calcium. The latter, through binding to calmodulin stimulates calcineurin provoking the dephosphorylation of NFAT and its translocation to the nucleus. Triggering of the Ras/Rac pathways results in the formation of an active AP-1 complex in the nucleus. Together, NFAT and AP-1 bind to consensus regions of the IL2 gene promoter and induce its transcription.

The* indicate guanine nucleotide exchange factors critical for Ras activation.

B: In presence of CSA or FK506, the multimeric complexx formed of CSA, cyclophilin and calcineurin inhibits the phosphatase activity of calcineurin. NFAT is thus sequestered in the cytosol and the IL2 gene remains silent.

complex formed of FK506 and a member of the family of the FK506-binding proteins (FKBP) associates also with calcineurin. The cyclophilins and the FKBPs are generally termed immunophilins. CSA and FK506 bound to their respective immunophilins prevent the translocation of the dephosphorylated form of NFAT to the nucleus, by inhibiting calcineurin activity.

A schematic view of the main actors involved in T-cell activation and the site of CSA action are summarized in (Fig. **1**a) and (**1**b), respectively.

A RE-EVALUATION OF THE MODE OF ACTION OF THE CSA AND FK506 : A STORY OF GENE SILENCING AND UP-REGULATION

For a long time it has been thought that the unique effect of the immunosuppressors on T-cells was to inhibit the transcription of genes encoding for cell growth and-or differentiation. As summarized by Kiani *et al.* [32], CSA inhibits the expression of the genes encoding for IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, GM- CSF and IFN-γ, the cell surface receptors CD40L, FasL, and IL-2Rα (CD25) and the transcription factor EGR3 in T cells. CSA blocks CD5 and Igk expression in B cells and of IL-4, IL-5, and TNF-α in mast cells and TNF-α and GM-CSF in NK cells. However, observations collected from a large number of laboratories suggested the existence of CSA-sensitive and -resistant pathways. This notion was established well before the characterization of the target of CSA (reviewed in [20]). At the cellular level, differences in susceptibility of naive and memory T cells to CSA were reported and further studies revealed that CSA does not prevent the induction of memory T-cells, giving a plausible explanation to the rapid graft rejection observed upon discontinuation of CSA therapy [33, 34, 35, 36].

Recently, researches in different fields of cell biology have shown that CSA and FK506 may augment activation-induced production of regulatory lymphokines and other immunological-relevant molecules. The cells studied were of different tissular origin and in various activation states. The constant finding that CSA and FK506 upregulate gene expression strongly suggests that the drug have a ubiquitous target. A general consensus is emerging that immunosuppressors therapy can, in addition to their well-established inhibition of cytokine genes, specifically stimulate the transcription of a gene or a set of genes that may play a role in the onset of the complications arising from their utilization. The collected data have opened an important new field of investigations.

CSA-DEPENDENT GENE INDUCTION: NOT ONLY AN ISSUE OF CELLS INVOLVED IN THE IMMUNE RESPONSE

Effect of CSA on Lymphoid Cell Activation

It is well substantiated that T-cell activation results in the novel expression of a large number of genes, while others are silenced. This phenomenon has a temporal component, since gene transcription is highly coordinated to ensure the acquisition of optimal effector functions and cell survival. Genes encoding for many protooncogenes are immediately induced, whereas genes encoding for cell growth and differentiation are induced later; finally a set of genes implicated in cell division are transcribed at a later time.

Of the genes implicated in the immune response, the ones encoding for many cytokines are blocked by CSA or FK506. Recent experimentations have addressed to the capacity of the immunosuppressors to prevent the transcription of other genes, also required in the immune response. Of the newly synthesized proteins, the earliest to be detected on cell surface is CD69 a molecule that it is likely to play pleiotropic immune regulatory functions in the activation and differentiation of a wide variety of hematopoietic cells [37]. CD69 is expressed by most T-cells at 24 hours following activation and disappears thereafter. In presence of CSA, the majority of the T cells express CD69, although the density at the membrane is 2-3-fold lower and CD69 mRNA accumulates at a lower level compared to activated control cells [38]. These data suggest that CD69 gene is regulated by CSA-sensitive and insensitive pathways. This hypothesis is substantiated by the report that AP-1, a CSA-resistant transactivator, is one of the elements involved in the regulation of CD69 expression [39] and by the finding that calcineurin, a CSA-sensitive element, is also involved in CD69 gene activation [40].

Among the surface markers that delineate T-cell subpopulations, the augmented expression of CD44 is associated to effector and memory cells (reviewed in [41]). CD44, a type I glycoprotein [42], is expressed late during T-cell activation. CSA does not affect the percentage of CD44 positive T-lymphocytes or the surface density following activation [38]. The regulation of CD44 gene is not yet known. It can be however deduced that its regulatory region does not include CSA-sensitive elements, *i.e.*, NFAT, and that CD44 expression is not driven by cytokines, as these molecules are not synthesized in presence of CSA.

One of the early reports that CSA may up-regulate gene expression was published in 1991. Ly-6E, a surface protein involved in T-cell activation, is induced by IFN-γ. Analyzing the mechanisms of Ly-6E expression in a T-cell lymphoma line (YAC-1), Altmeyer *et al.* showed that this antigen was superinduced when CSA or FK506 were concomitantly added to IFN-γ [43]. Both molecules were active at pharmacologically concentrations, similar to those inhibiting normal T-cell activation, suggesting that comparable mechanisms are involved. However, CSA increased the sensitivity of signaling by IFN-γ receptors. This report was the first of a set of studies analyzing whether CSA (or FK506) may up-regulate gene expression in T-cells when activation is achieved by TCR engagement or upon co-ligation of TCR and costimulatory molecules.

TCR ligation increases the cellular level of LAT [44], a molecule critical for T-cell development and function [45]. Increase in LAT expression involves the serine/threonine kinases PKC and MEK. Strikingly, CSA and FK506 strongly potentiate TCR-induced LAT expression [44]. In

contrast, Ca^{2+} ionophores, which activate calcineurin by increasing intracellular Ca^{2+}, block the TCR-increased LAT expression and CSA and FK506 reversed the Ca^{2+} ionophores' inhibitory effect. These data indicate that although the inhibition of calcineurin by CSA and FK506 blocks certain aspects of TCR signaling important for cytokine gene expression, other features of TCR signaling, normally suppressed by calcineurin, are upregulated. This, in turn, might provoke the enhanced expression of other control signaling molecules.

Particular attention was paid to the effect of CSA on T-cell activation throughout CD28, a homodimeric glycoprotein expressed on the surface of most human and murine T cells and acting as a major costimulatory receptor (reviewed in [46]). CD28 amplifies TCR-mediated signaling and cytokine gene expression [47-49]. T-cell co-stimulation by anti CD28 mAbs results in the increase stability of IL2 mRNA and in its enhanced accumulation [50]. Depending on the mode of T-cell stimulation, increased IL2 mRNA

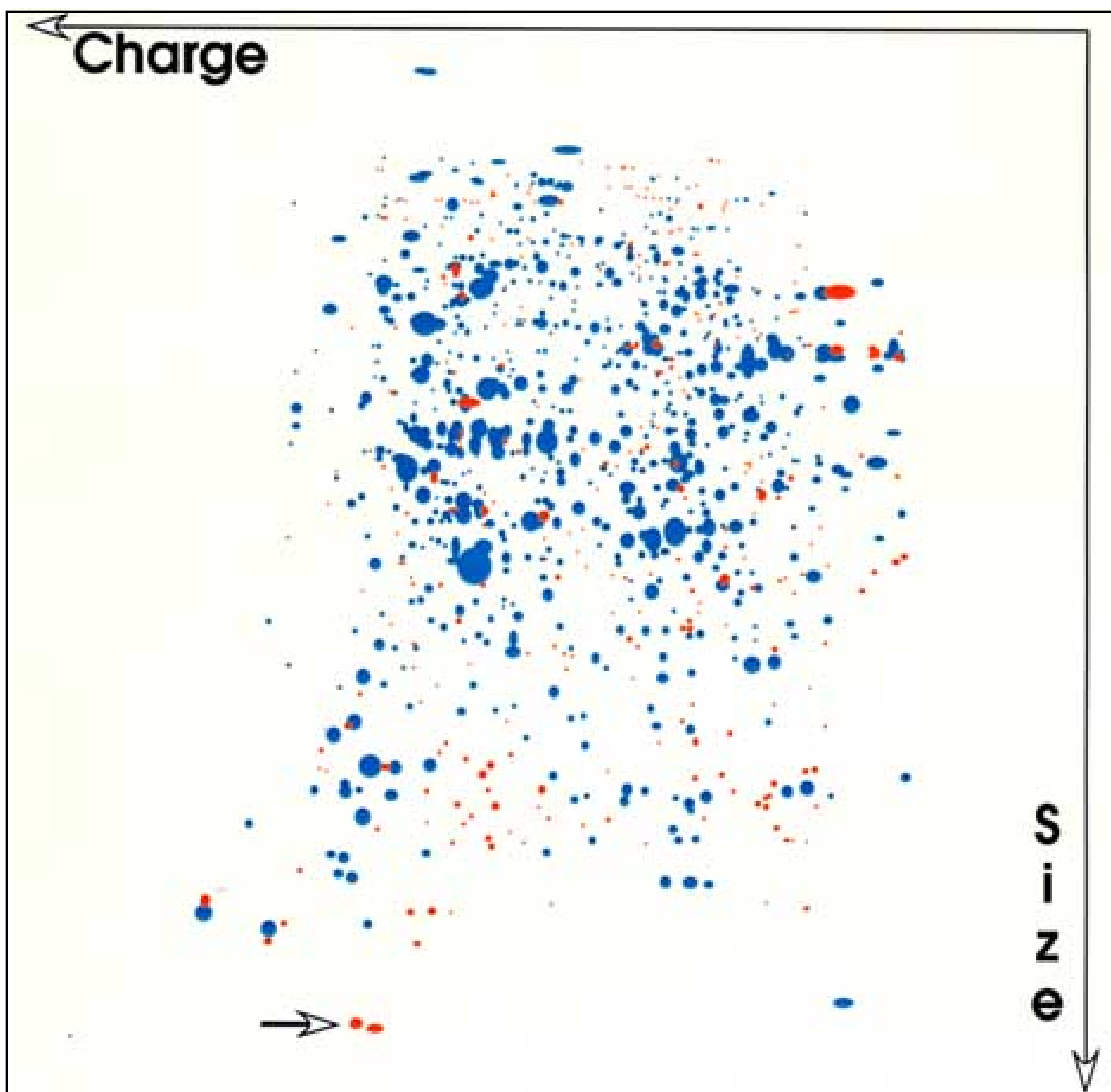

Fig. (2). Proteomic analysis of T-cell Ctivated in presence or absence of CSA: induction by the immunosuppressor of a large set of novel proteins

T-cell activated in presence and absence of CSA were labeled with ^{35}S-methionine and the proteins submitted to 2-D gels electrophoresis. The computer-modeled images of the gels were compared by a software system in order to assess the proteins present or absent in a given group. The red spots represent the proteins labeled exclusively in presence of CSA. The arrows identify spots found only in presence of CSA.

through CD28 has been shown to be resistant, in part, to CSA [50]. Notably, CSA does not to inhibit IL-2 mRNA accumulation following CD28 plus PMA stimulation [50]. In conclusion, being insensitive to CSA, CD28 costimulation is distinct from TCR signaling.

In addition, the study of the effect of CSA [51, 52] and FK506 [53] on CD28 ligation has also been rewarding because it raises the hypothesis that cytokine gene expression may be mediated by negative regulation. This point is exemplified by experiments analyzing IL-13 and IFN-γ gene induction. Stimulation of human T-cells by anti-CD3 and anti-CD28 mAbs results in the production of IL-13. Such a production is also found with a combination of PMA and anti-CD28 mAbs. However, additional ligation of the TCR by anti-CD3 mAbs produces a strong inhibition of IL-13 synthesis implying that TCR signaling modulates the expression of this cytokine [51]. The findings that CSA reverted the inhibitory effects of anti-CD3 mAbs and that it upregulates IL-13 synthesis indicates the existence of negative regulatory circuits. For IFN-γ, two studies demonstrated that CSA and FK506 did not block the transcription of this gene but that they even up-regulate its expression, when T-cells were activated by anti-CD3 and anti-CD28 mAbs [52, 53].

The general picture of CSA upregulating some gene transcription is comforted and extended by a proteomic approach in which new protein synthesis in T-cells activated in absence and presence of CSA were compared [38]. Quantitative and qualitative 2-D gel analyses were done on activated, CSA-untreated and treated, T-lymphocytes pulsed at different times with ^{35}S-methionine. Labeling was performed at early periods in order to assess the effect of CSA on genes expressed immediately following activation. The last pulse corresponded to a time when many of the T-cell-derived cytokines are synthesized. The comparison of each spot in congruence with its matched counterpart revealed that some proteins were made only in activated T-cells and that their number increased with time. This result was anticipated as many genes encoding cytokines are blocked by the immunosuppressor and that transcription of genes depending on IL-2 interaction with its receptor, genes that are involved in cell differentiation and division, are not transcribed in absence of IL-2. Also expected was the finding that many polypeptides were common to both activated and immunosuppressed T-cells. Remarkably, this study revealed that more than a hundred of proteins, not found in resting or activated T-cells, were induced when stimulation is carried out in presence of CSA (Fig. **2**). The identification of these proteins by representational difference analysis (RDA), currently under study in our laboratory, should allow ascertaining whether these genes can be implicated in the various complications resulting from CSA therapy.

Discontinuation of CSA Therapy Induces A Novel Program of Gene Expression

Discontinuation of CSA therapy results in a rapid graft rejection that shares the characteristics of a secondary immune response. Addressing to this result, we have found that elimination of CSA from T-cells polyclonaly activated in presence of the immunosuppressor had dramatic effects on gene expression [26, 54]. Particularly, IL-2 mRNA accumulation was observed demonstrating that the corresponding gene was transcribed without novel T-cell activation. This finding implied that both NFAT and AP-1 were present in the nucleus. This prediction was experimentally demonstrated: indeed NFAT translocates to the nucleus as soon as CSA is withdrawal and AP-1 molecules persisted in the nucleus for at least 48 hours. This contrast with the rapid decay (an half-life of 1-2 hours half-life) observed in absence of CSA [26]. AP-1 is responsible, either by itself or in conjunction with other transcription factors, for the activation of several genes [55]. Thus, the persistence of AP-1 confers to T-lymphocytes activated in the presence of CSA particular features that distinguish them from both naive and primed T-lymphocytes [26].

An analysis of the proteins newly synthesized following CSA withdrawal revealed both qualitative and quantitative changes in protein abundance [54]. The proteomic analyses substantiate and extend previous research on IL-2 gene expression to hundreds of polypeptides. Around 200 polypeptides, not previously detected in T-cells activated in absence of CSA nor in cells activated in presence of CSA, are biosynthesized upon immunosuppressor withdrawal. This analysis of the effect of CSA on gene expression may have implication in understanding the mechanisms involved in graft rejection following the arrest of CSA therapy once these genes are identified. It remains also to be shown whether the changes in protein representation indicate a spontaneous reshuffling of cellular differentiation functions or a general shift towards a new cellular homeostasis.

Non-lymphoid Cells

Treatment of HeLa cells with low concentrations of CSA induced the synthesis of GRP78, a stress protein [56]. In a subsequent study, the same group reported that CSA increased the expression of the heat shock protein HSP27 and of two heat shock transcription factors, HSF1 and HSF2 [57]. Remarkably, FK506 had no detectable effects. Still, the two immunosuppressors have similar mode of action when tested on activated cells implying that these molecules might have different targets in resting and activated cells. The finding that CSA increased p53 induction in a kidney epithelial cell line raises the possibility that calcineurin is not the unique target of CSA and that this still unknown pathway might be involved for nephrotoxicity and tumor formation [57].

A prominent side effect of CSA administration is gingival overgrowth (reviewed in [58]). This overgrowth does not develop in all patients and appears within 1 to 3 months after initiation of CSA therapy. Different groups investigated the possibility that the immunosuppressor altered fibroblast activity in the connective tissue within the periodontium, either directly or by interacting with macrophages. Two growth factors, platelet-derived growth factor (PDGF) and transforming growth factors (TGF-β) are the major elements in wound repairing and connective tissue homeostasis. PDGF mRNA is increased in overgrown tissues from CSA-treated patients and is produced by mature

macrophages [59]. Gingival tissues of patients receiving CSA therapy and exhibiting gingival overgrowth have an increased levels of IL-6 protein and IL-6 mRNA [60]. Further analysis lead to the conclusion that the gingival fibroblasts are the target of CSA and the source of IL-6 production [61]. The heterogeneity of the cells makes the mechanism of gingival overgrowth by CSA therapy difficult to analyze. Nevertheless, all the data in the literature strongly suggest that the effect of CSA on gingival growth are multifactorial and direct the over-expression of a set of genes involved in wound repairing and connective tissue homeostasis.

CSA THERAPY AND TGF-Β PRODUCTION, A TRANSFORMED VIEW OF CSA EFFECTS

TGF-βs are a group of multifunctional growth factors, which inhibit or promote cell cycle progression in various cell types. The discovery that, in contrast to IL-2, CSA and FK506 increased the level of TGF-β [62, 63] may explain the efficiency of these immunosuppressors. It can be postulated that the impairment of IL-2 production and increased TGF-β accumulation reinforce each other resulting in the complete arrest of T-cell division and differentiation.

Cell cycle arrest has been partially attributed to the regulatory effects of TGF-β on both the levels and the activities of the G1 cyclins and their kinase partners. Particularly, TGF-β induces the p21 protein, the cyclin inhibitor [64]. Further analysis revealed that three interconnected pathways mediate TGF-β-receptor signaling. The finding that the ubiquitous FK506-binding protein FKBP12, in addition of being a subunit of two intracellular calcium release channels, is also a subunit of the TGF-β receptor has important consequences [65]. Disruption of FKBP12 leads to over activation of p21 and to a decreased cell division rate [66] suggesting that it acts as negative regulator of the TGF-β-receptor signaling. FK506 binding to FKB12 may also result in a comparable inactivation of FKB12, directly inducing p21 synthesis. Alternatively, up-regulation of p21 may also be achieved by TGF-β signaling through its receptor.

TGF-β has been implicated in the pathogenesis of wound healing and scarring. The finding that CSA increased the production of TGF-β in epithelial cells suggested a potential mechanism for some of the common complications associated with CSA therapy. To the CSA-dependent increased production of TGF-β in activated T lymphocytes corresponds an enhanced expression of TGF-β receptor [67].

CSA and FK506 therapy have been associated with cancer progression. The high incidence of neoplasm and its aggressive evolution are thought to be due to the resulting impairment of the organ recipient's immune-surveillance system [68]. However, Hojo and colleagues have recently proposed a mechanism for the amplified malignancy that is independent of host immunity [69]. They took advantage of the finding that TGF-β can promote the invasion and metastasis of adenocarcinoma cells [70]. A non-transformed human pulmonary adenocarcinoma cell line, which growth and functions are regulated by TGF-β and expressing TGF-β receptors was used to test the hypothesis that CSA can induce an invasive phenotype. These cells respond to CSA in a concentration-dependent manner by an increased expression of TGF-β gene. Exposure of these cells to CSA, *in vitro*, results in a change of shape, in increased mobility and, unlike normal cells, in their capacity to grow without being anchored to a solid surface. CSA can also induce morphological and functional alterations in other cell types as murine renal cell adenocarcinoma cells, mouse mammary

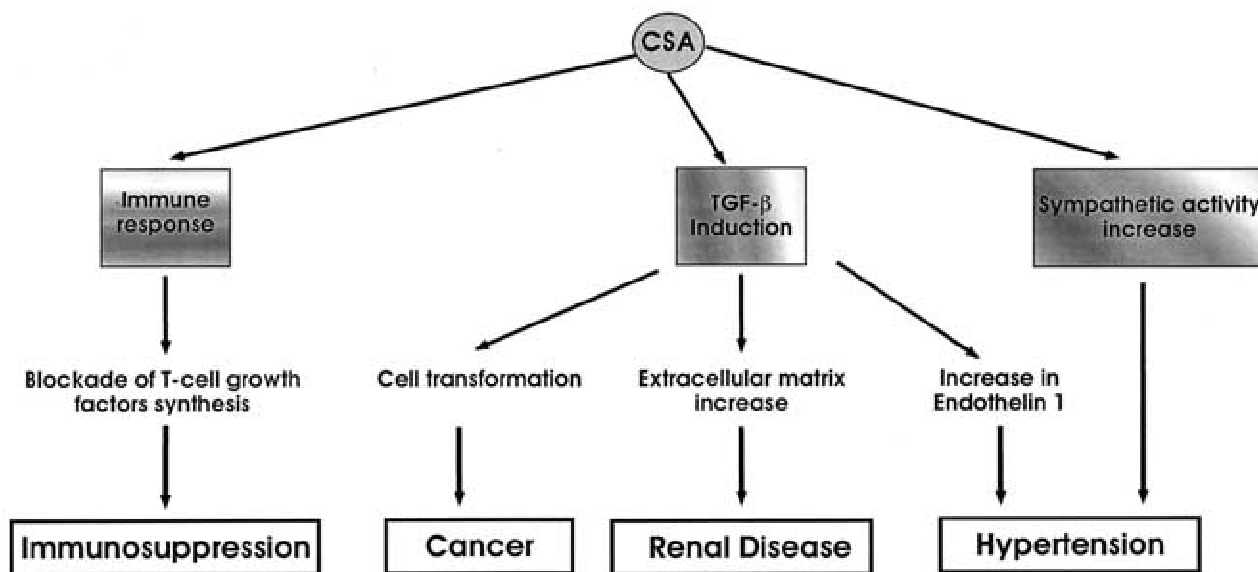

Fig. (3). Linkage between the immmunosuppressive properties of CSA and FK506 and the sides effects induced by the two molecules.

CSA and FK506 induce immunosuppression by blocking the transcription of genes encoding for T-cell growth and differentiation factors. By inducing TGF-β, CSA and FK506 facilitate cancer cell progression, renal diseases and hypertension.

gland epithelial cells or mink lung epithelial cells. That mAbs against TGF-β could suppress this newly acquired phenotype clearly demonstrate that this molecule mediates such a process, either directly or indirectly. This conclusion was reinforced by *in vivo* studies. In immune-deficient SCID-beige mice, receiving different types of tumor cells, more secondary tumors developed in the lungs in the presence than in the absence of CSA. The use of these mice minimizes the possibility that CSA-induced depression of the host's immune system contributes to tumor progression.

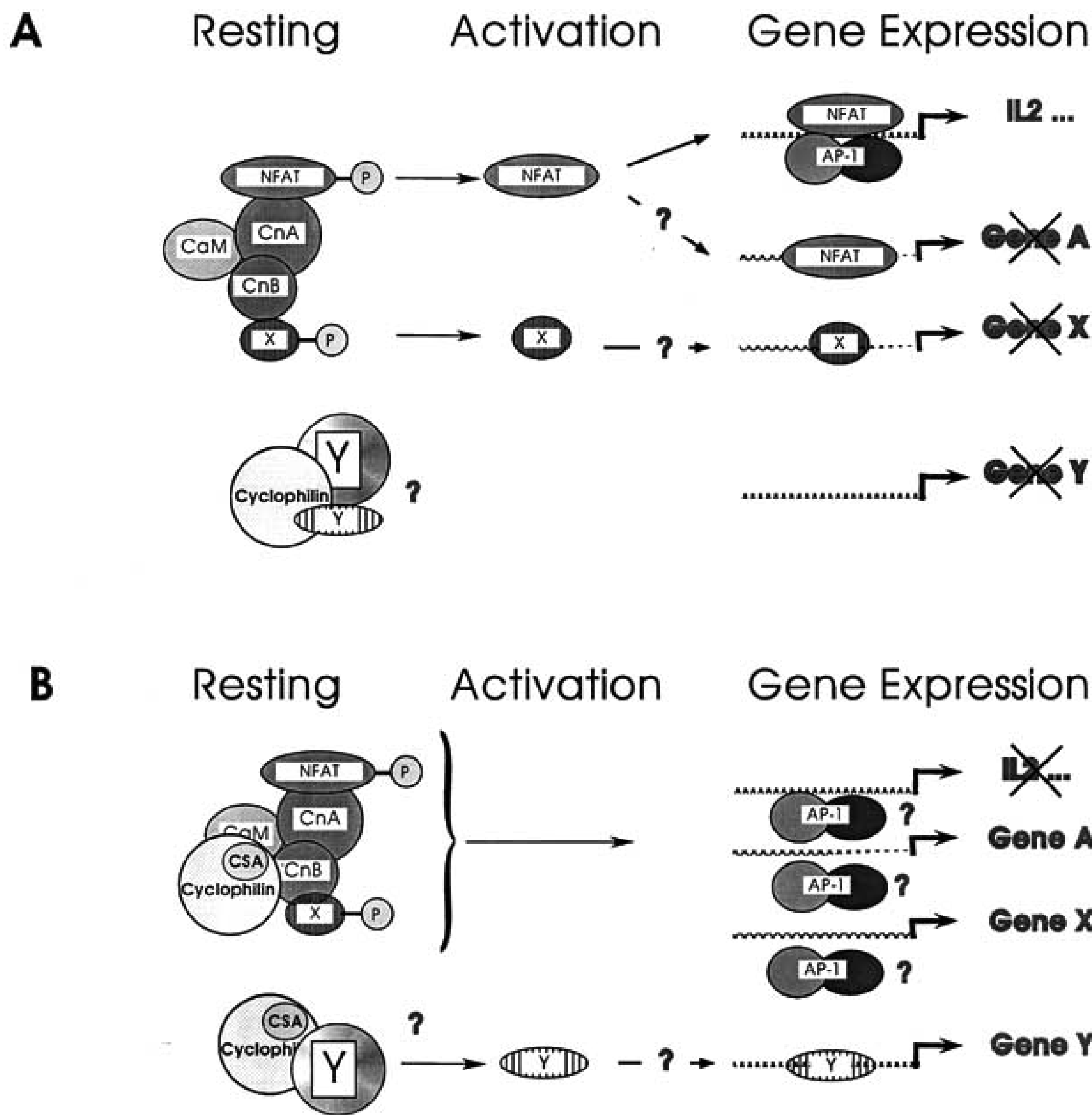

Fig. (4). Known and hypothetical pathways linking gene silencing and exxpression to CSA immunosuppressive activities.

A: Dephosphorylation of NFAT and its translocation to the nucleus results in the exxpression of the IL2 gene.In contrast, binding of NFAT to the promoter regin of gene A results in its silencing . A hypothetical transcription factor "x" is also dephosphorylated by calcineurin and upon translocation to the nucleus blocks the expression of gene "X". A complex of cyclophilin and a hypothetcal "Y" protein retains in the cytosol a transcription factor "y", essential for the exxpression of gene "Y".

B: By bloking the calcineurin activity, CSA and FK506 keep NFAT and the hypothetical transcription factor "x" in the cytosol allowing for the expression of the gene A and "X". The supposed complex between CSA or FK506 and cyclophilin liberates the hypothetical transcription factor "y" resulting in the transcription of the gene "Y".

Anti-TGF-β mAbs prevent the CSA-induced increase in the number of metastases and stopped, *in vitro*, the CSA-induced phenotypic changes of the adenocarcinoma cells. These data suggest that CSA can promote cancer progression by a direct cellular effect that is independent of its effect on the host's immune cells, and that CSA-induced TGF-β production is involved in this process [69]. It should be however stressed that although the mechanisms by which CSA induces secondary cancers may be explained by this process, nothing is known on the effect of the immunosuppressor in converting cells from a benign to cancerous state.

In Figure **3**, we propose a model, inspired by the one presented by M. Suthanthiran [71, 72], linking CSA to immunosuppression, fibrosis, hypertension and cancer.

CONCLUSION

CSA was a critical instrument in assessing the pathway(s) linking the membrane biochemical events induced by TCR occupancy to nuclear processes culminating in gene expression. The role of calcineurin, as a key serine/threonine phosphatase, and of NFAT, as a main inducer of gene expression, are now well established. Immunosuppression is directly linked to the blockade of calcineurin activity and to the defect of T-cells to transcribe genes encoding for growth and differentiation factors. The complementary picture emerging is that CSA and FK506 are also acting at other sites downstream of TCR signaling and that both drugs can provoke or increase the transcription of various genes. The molecular mechanism(s) by which calcineurin inhibition by CSA and FK506 leads to an increased gene transcription have not yet been identified but testable predictions can be made.

In figure 4 (Fig. **4A** and **4B**) are represented the known biochemical pathways that are targeted by CSA and FK506 and hypothetical events that may explain how these molecules activate alternative biochemical pathways that culminate in a new program of gene expression. Some of these pathways are identical to the one provoking cytokine gene expression, but the known transactivator NFAT has a negative effect on gene expression. Alternatively, substrates or calcineurin inhibitor are implicated. Finally, a particular pathway linking cyclophilin to a yet unidentified transactivator is activated by TCR occupancy.

Gene transcription is modulated by molecules that bind to regulatory regions located primarily at the 5' end of a gene. Negative and positive transactivators participate in this regulation. Although originally described as T-cell-dependent transcription factors, evidence is emerging that NFATs control processes of cell differentiation in progenitors of multiple lineages. The NFAT family is composed of 5 members: NFAT1, -2, -3, -4 and -5/TonEBP (reviewed in [32, 73]). In T-cells, NFAT1 is known to act as a positive regulator for cytokine gene transcription. Recent results by Ranger *et al.*, have shown that NFAT1 is a repressor of chondrogenesis [74]. In keeping with this observation, it may be hypothesized that, in addition to its positive regulatory activity, one of the NFATs may act as a negative element for the active transcription of a gene, or a given set of genes, in T-cells. Nuclear translocation and binding to their consensus regulatory sequence, either alone or in combination with other transactivators will result in gene silencing. The blockade of calcineurin activity by CSA will result in the retention of this NFAT into the cytosol. Since AP-1 is induced in presence of CSA, it may postulated that it will allow the expression of the corresponding gene.

Calcineurin has other substrates than NFAT. It binds to and dephophosphorylates the transcription factor Elk-1 [75] and regulates NFκB [76] and CREB activities [77]. Calcineurin cooperates with PKC-θ to activate JNK, which activates c-Jun and ATF-2 transactivators [78]. These examples link calcineurin activation to different biochemical pathways that are generally associated to gene up-regulation. However a negative role of calcineurin have also been described. Among the other proteins binding to calcineurin, Cabin-1/Cain-1, was shown to be a potent calcineurin inhibitor, but not a substrate, and may serve as a negative regulator of TCR signaling via inhibition of the phosphatase activity [79, 80]. Data on the effects of the CSA and FK506 on concomitant CD3/CD28 ligation also indicate that activation of calcineurin has inhibitory effects on some gene expression, an inhibition that can be released by the immunosuppressors. The question should be asked whether one of the calcineurin substrates, a phosphorylated transactivator, once dephosphorylated and translocated to the nucleus, invalidates gene expression. The blockade of calcineurin activity by the immunosuppressors will provoke its retention in the cytosol inducing the expression of a gene or set of genes sharing similar control elements. Finally, an unidentified pathway, in which cyclophilin retains a transactivator in the cytosol is set in motion once CSA has bound to its receptor provoking the translocation to the nucleus and gene transcription.

The recent finding that calcineurin activation have conflicting effects on gene expression is changing our understanding of the mode of action of the immunosuppressors. The hypothesis that CSA and FK506 may have other targets than calcineurin and that calcineurin activation may also results in gene inactivation opens new horizons for a more rational approach to an efficient transplantation. These new findings are important in understanding the mechanisms linked to the toxicity and the tumor promoting properties of the immunosuppressors and help to design more specific drugs.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. Fréderique Michel for helpful discussions and suggestions. The Institut Pasteur and the CNRS supported this work.

ABBREVIATIONS

AP-1 = Activator protein-1

CSA = Cyclosporin A

FKBP = FK506-binding protein

GTPases = GTP Phosphohydrolases

IL- = Interleukin

LAT = Linker for activation of T cells

MAPK = Mitogen-activated protein kinases

MHC = Major histocompatibility complex

NFAT = Nuclear factor of activated T-cells

PKC = Protein kinase C

PDGF = Platelet-derived growth factor

PLC = Phospholipase C

PTK = Protein tyrosine kinase

TCR = T-cell receptor

TGF-β = Transforming growth factor

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