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Transcription Factor Profiling Shows New Ways Towards New Treatment Options of Cutaneous T cell lymphomas

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Abstract: Most oncogenes encode activators of transcription factors or transcription factors themselves. Transcription factors that are induced by growth stimuli are, in contrast to transcription factors that regulate house keeping genes, tightly regulated and only active, when a stimulus (e.g. cytokines or other growth factors) is given. Examples of such transcription factors are members of the jun, fos, myc, NFkB and STAT gene families. In cancer cells this regulation is interrupted, resulting in constitutive activities of transcription factors that are normally silent. This in turn results in the increased expression of target genes that are necessary for growth and protection from apoptosis. Since inducible transcription factors are activated by specific pathways, the identification of unusual constitutively active transcription factors also identifies the involved signal transduction pathway. Inhibitors of the components of these pathways may be effective anti-cancer agents, as they interrupt the abnormal signalling and in cancer cells. We applied this strategy for two forms of cutaneous T cell lymphomas and identified several groups of agents that may be the prototypes of new drugs to fight these diseases.

Key Words: Signal transduction, tyrosine kinase inhibitors, apoptosis inducers, skin cancer, sodium salicylate, arsenic trioxide, potassium antimonyl tartrate.

CUTANEOUS T CELL LYMPHOMAS

Cutaneous T cell lymphomas (CTCL) comprise a spectrum of lymphoproliferative disorders of the skin [1]. The most frequent forms are mycosis fungoides (MF) and its leukemic counterpart, the Sézary syndrome (SS). MF evolves through several clinical stages (patch, plaque, and tumor stage) and leads ultimately to death often caused by rapidly growing and ulcerating tumours as well as by immune disorders. Lymph nodes are infested in later stages and infestation of inner organs is rare. The median survival time of MF patients is 8-20 years. Characteristics of the Sézary syndrome are generalised erythroderma, leukemic T cells in the blood and a shorter life expectancy (average 3 years). This is probably due to the circulating malignant T cells, which produce cytotoxic T cells, suppressing interleukins like IL-10 in high amounts. At the moment there is no therapy to cure both forms of CTCL. In early stages CTCL is often misdiagnosed as eczema or psoriasis.

MF patients in early stages are generally treated with glucocorticoids or more effectively with psoralen and subsequent UVA treatment (PUVA). PUVA therapy shows complete remission for 6-30 months in more than 80% of the patients. However all patients relapse. Advanced stages and Sézary syndrome are treated by PUVA plus interferon alpha, extracorporal photopheresis, total skin electron beam irradiation or chemotherapy (e.g. low dose methotrexate, chlorambucil plus prednisone, retinoids, interferon alpha). All these treatments lead to partial or total remission, but not to a final cure of the disease [2].

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KINASES AND TRANSCRIPTION FACTORS IN CANCEROGENESIS

Cancerogenesis and cancer progression involve mutations of several oncogenes and tumor suppressor genes [3]. Many oncogenes code for molecules that are involved in signal transduction, these include growth factor receptors (e.g. EGF, PDGF receptors), tyrosine kinases (Jak, c-abl, c-src), serine/threonine kinases (raf, c-mos) and transcription factors (c-jun, c-fos, c-rel). The first step of a typical signal transduction pathway is that a growth factor binds to its receptor on the cell membrane. This leads to changes of the molecular structure of the receptor molecule (e.g. EGF, PDGF receptor), and the activation of a tyrosine kinase, which is part of the receptor molecule. In the case that the receptor does not possess a tyrosine kinase activity it can recruit directly or via adaptor molecules free tyrosine kinases to the receptor/ligand complex (e.g. Jak, c-abl, c-src). The activated tyrosine kinases then activate serine/threonine kinases, which activate transcription factors by phosphorylation. These activated transcription factors are now able to move to the nucleus and to bind to their target sequences on the DNA. Here they help to recruit co-factors of RNA polymerase to the promoter and to form an active transcription complex. This scheme has many variations e.g. some tyrosine kinases can directly activate transcription factors and sometimes a whole cascade of serine/threonine kinases (MAPKKK pathways) may be required for transcription factor activation [3].

In cancer mutations of signalling transducing molecules (receptors, tyrosine kinases, serine/threonine kinases, transcription factors) can mimic a stage of permanent stimulation. The replacement of a tyrosine residue in tyrosine kinases, which often phosphorylate themselves, by an acidic amino acid (glutamic acid, aspartic acid) can lead to a per-

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manently active tyrosine kinase molecule, since the negative charge of the acidic amino acid mimics the negative charge, which is normally introduced by the addition of a phosphate molecule to the tyrosine residue. Such a mutant would constitutively activate transcription factors, which in turn can activate genes that are necessary for proliferation and cell survival (e.g. bcl-2). Constitutive tyrosine kinase can also be achieved by the loss of a regulatory domain of the enzyme by deletion or missense mutants. The same types of mutations can also occur in serine/threonine kinases and transcription factors.

Every signalling pathway activates only a certain subsets of transcription factors. Thus it is possible to identify a disturbed signalling pathway by the determination of abnormal constitutive transcription activities in a cancer cell. There are inhibitors of tyrosine kinases, serine/threonine kinases and transcription factors, which can serve as prototypes of future anti-cancer drugs. One example is Imatinib (Gleevec, STI157), which inhibits the tyrosine kinases c-abl and c-kit. It is effective against chronic myeloic leukemia (CML) [4] and gastro-intestinal stromal tumors (GIST) [5].

NFkB TRANSCRIPTION FACTOR ACTIVITIES IN MYCOSIS FUNGOIDES AND SÉZARY SYNDROME CELLS

To profile mycosis fungoides and the Sézary syndrome, first the NFkB transcription factor family was analyzed. A screening of various B and T cell lymphomas [6] had shown that 10-15% of CTCLs and the SS cell line HUT78 contain a chromosomal translocation of the NFKB2 (lyt-10) gene, which codes for the transcription factor NFKB2 [7, 8], also called p52. HUT 78 cells produce a truncated NFKB2 protein that is constitutively located in the nucleus [9] since it lacks large parts of its regulatory region that retains it in the cytoplasm and thus prevents it to move into the nucleus and to bind to its target sequences on the DNA. This modified transcription factor therefore may act like other oncogenes (e.g. v-jun, v-Rel) as a permanently active transcription factor, which disturbs normal gene expression.

NFKB2 belongs to the rel/dorsal/IkB superfamily of transcription factors, which consists of three groups [10, 11]. Group I consists of proteins of about 65 kD (cRel, RelA, RelB) containing the rel-domain, which is necessary for DNA binding, activation of transcription and interaction with other members of the rel/dorsal/IkB-superfamily. Group II consists of the genes NFKB1 and NFKB2, which code for proteins of 105 and 100 KD respectively (p105 and p100). They contain a rel domain, an acidic region, and seven ankyrin repeats that tether these proteins to the cell membrane. The group II proteins p105 and p100 are processed by a protease into the transcriptionally active proteins p50 (NFKB1) and p52 (NFKB2) respectively, which lack the ankyrin repeats. The activity of the p100 processing protease depends on the activity of the IkB kinase alpha (IKK alpha) [12]. Group III consists of the IkB proteins, which also contain seven ankyrin repeats and an acidic region, and resemble the carboxyl terminal parts of p105 and p100 proteins, which are cut off by a protease.

The p50/p65 and p52/RelB proteins respectively form heterodimers that migrate into the nucleus, bind to NFkB sites on the DNA, and activate gene transcription. Group II proteins (p50 and p52) can also form homodimers that also bind to the NFkB sites. They act generally as transcriptional repressors, but act sometimes as transcriptional activators, depending on the context of transcription factor binding sites in a given promoter or enhancer, and on the presence of cell specific co-factors [13].

The IkB proteins (group III) bind to group I/II heterodimers and sequester them to the cytoplasm, blocking thus the activation of gene transcription by NFkB. When an NFkB activating stimulus is given IkB proteins are phosphorylated by the IkB kinase beta (IKK beta) complex and subsequently ubiquitinylated and degraded [14-20].

An exception amongst the IkB proteins is Bcl-3. It forms p52/p52/Bcl-3 heterotrimers with p52 [21], which bind to NFkB sites and activate gene transcription. Bcl-3 has also been reported to activate transcription by the removal of p50/p50 homodimers from NFkB binding sites [22].

The NFkB binding sites normally are not occupied by rel/dorsal/IkB proteins [10, 11]. Binding to NFkB sites is induced or enhanced by activators of the protein kinases A and C (IL-1, TNF, caffeine, lectins and phorbol esters), viruses (HIV, CMV) and oxidants (H_2O_2, O_2 radicals), which initiate the phosphorylation and degradation of IkBs.

A NFKB1 or NFKB2 protein, whose carboxyterminal ankyrin repeats have been deleted cannot be retained in the cytoplasm it has the potential to constitutively activate gene expression and to disturb the normal gene expression pattern in T cells. Thus cytokine and cytokine receptor genes (IL-2, IL-6, IL-15, TNFalpha, GM-CSF, G-CSF, IL-2R alpha) that contain NFkB sites can be constitutively activated and cause cell proliferation.

To profile mycosis fungoides and the Sézary syndrome nuclear extracts of mycosis fungoides and the Sézary syndrome cell lines were analyzed by electrophoretic mobility shift assays (EMSA) and transcription factor ELISA (TF-ELISA). Surprisingly all five members of the NFkB gene family: p50, p52 (group I), p65, RelB, and c-Rel (group II) were constitutively active in the tested MF and SS cell lines. Western blot analysis of the nuclear extracts confirmed that all NFkB proteins were of normal size with the exception of the p52 protein of the SS cell line HUT78, which carries an NFkB2 translocation. The Western blots also detected the Bcl-3 protein in the nuclear extracts of the tested MF and SS cell lines.

The immunohistochemical analysis of skin lesions of MF and SS patients with specific antibodies shows that p50 and Bcl-3 were present in the nuclei of malignant cells at the earliest stages of these diseases. The proteins p65, p52 and RelB were also found in earlier stage, but a simultaneous expression of p52 and RelB occurred only in the tumor stage. The c-Rel protein was only found in some tumor stage samples [23-25]. These findings indicate that the MF and SS cell lines mirror the tumor stage of MF and SS.

STAT TRANSCRIPTION FACTOR ACTIVITIES IN MYCOSIS FUNGOIDES AND SÉZARY SYNDROME CELLS

Interleukin 7 (IL-7) and interleukin 15 (IL-15 have been found to be growth factors for MF and SS cells, [26, 27]. Since interleukins signal through the Jak and Tyk tyrosine kinases, one can assume that the STAT (signal transducer and activator of transcription) transcription factors, which are directly phosphorylated and activated by Jak and Tyk tyrosine kinases, are involved in the pathogenesis of MF and SS. The test of the IL-7 and IL-15 independent MF and SS cell lines MyLa 2059 and HUT78 indeed detected constitutive DNA-binding of the STAT proteins 2, 3, 5 and 6 in the nuclear extracts of these cell lines. In the IL-7 and IL-15 dependent SS cell line SeAx however, the binding activities of these 4 STAT proteins were dependent on the presence of these two growth factors in the medium. All these four STAT proteins were of the expected size, indicating that no translocations of these transcription factors has occurred. The fact that the MyLa 2059 and HUT78 have become IL-7 and IL-15 independent can therefore be explained by the assumption that a mutated or otherwise activated tyrosine kinase has become constitutively active in these cells (see below).

In the biopsies of MF and SS patients activated STAT5 was already detected in early stages, whereas STAT2, 3, and 6 become active only in the tumor stage [28, 29]. Recently it was reported that STAT3 is also already active in early stages [30]. These activities may be triggered by tyrosine kinases that are IL-7 and IL15 independent or tyrosine kinases that are activated by IL-7 and IL-15 produced by skin keratinocytes and fibroblasts or the MF and SS cells themselves [27].

CONSTITUTIVELY ACTIVE TRANSCRIPTION FACTORS OF THE JUN/FOS, MYC/MAX AND MYB GENE FAMILIES IN MYCOSIS FUNGOIDES AND SÉZARY SYNDROME CELLS

MF and SS cell lines and skin lesions were also tested for constitutively active members of the jun/fos, myc/max and myb gene families. For the jun/fos family no DNA binding activities could be found for the proteins c-Fos, Fra-1, Fra-2, and c-Jun, however constitutive DNA binding activities were reported for JunB and JunD. Constitutive DNA binding was also observed for the myc/max and myb gene family members c-Myc, Max, and c-Myb. No binding was observed for N-myc and the mad proteins. The DNA-binding of JunD and c-myb was dependent on IL-7 and IL-15 in SeAx cells, whereas the binding of c-Myc/Max heterodimer was independent of IL-7 and IL-15 [31]. In all three tested MF and SS cell lines two other IL-7 and IL-15 dependent DNA binding proteins were found, which bound like the c-Myc/Max heterodimer to the so called E-box DNA element, but did not react with c-Myc and Max antibodies. These complexes may contain the protein USF-1 and USF-2, since it has been reported that these proteins bind to the same recognition sequence as c-Myc/Max heterodimers. The c-Myb protein was detected in the MF and SS cell lines and skin biopsies. It could already be detected in early stage skin lesions [32].

DETERMINATION OF THE INVOLVED SIGNALLING PATHWAYS

Several substances are known to inhibit NFkB, STAT and Fos/Jun DNA-binding. Sodium salicylate is a potent inhibitor of the IKK beta (inhibitor of kappa B kinase beta) kinase. This kinase phosphorylates inhibitor of kappa B (IkB) proteins at a serine residue and targets it for proteolytic degradation. The destruction of the IkBs allows the NFkB molecules to enter the nucleus and to activate transcription. The addition of sodium Salicylate to MF and SS cell line led to a reduction of the constitutive NFkB binding and at longer incubation to the apoptosis of these cells. This experiment showed that constitutively active IKK beta activity is necessary for the maintenance of constitutive NFkB activities. The addition of the src-type tyrosine kinase inhibitor herbimycin A had the same effects, indicating that a src-type tyrosine kinase may be involved in NFkB and IKK beta regulation, since the prototype of these kinases, c-src, has been reported to phosphorylate IKK beta [33]. An immuno-histochemical screening of MF ands tumors with specific antibodies showed that c-src and the related c-yes tyrosine kinase are expressed in these tumors. Interestingly c-src is also involved in the activation of STAT transcription factors. The expression of c-yes increased strongly in the tumor stage. C-Src was already found in the early stages, however its activated (i.e. phosphorylated) form was only found in the tumor stage. Therefore one can assume that interleukin-dependent tyrosine kinases are responsible for the phosphorylation of NFkB and STAT protein in the early stages, whereas the interleukin-independent c-Src kinase takes over this job in the tumor stage. The immunohistochemical stainings also revealed the expression of the tec-type tyrosine kinase bmx [34], which has also been implicated in the activation of several STAT molecules [35]. Sodium salicylate and herbimycin A could therefore be prototypes for novel anti-cancer drugs against MF and SS. In contrast to these two agents the NFkB and jun/fos antagonist dexamethasone had only a transient effect on the IL-7 and IL-15 dependent SS cell line SeAx. PP1, an inhibitor of the T cell receptor associated tyrosine kinases lck and fyn inhibited the constitutive NFkB activities, but had no proliferation inhibiting or cell killing effects, indicating that sodium salicylate and herbimycin A may have more targets than PP1 [36].

The IKK complex has also been reported to have an influence on the cell cycle. IKK alpha has been reported to suppress the cell cycle regulator p27 by the activation of p52/RelB heterodimers that activate the skp2 gene. The Skp2 protein targets p27 for ubiquitin-dependent degradation [37]. It is capable to inactivate RB by hyperphosphorylation [38] and thus to increase cell cycle progression and proliferation. Since RB is hyperphosphorylated in MF and SS cell lines and skin lesions [39], this could be another explanation of this finding, besides the already described inhibition of the p16 tumor suppressor gene [39], which controls the phosphorylation of RB by cyclin dependent kinases. An overview of these pathways is given in Fig. (**1**).

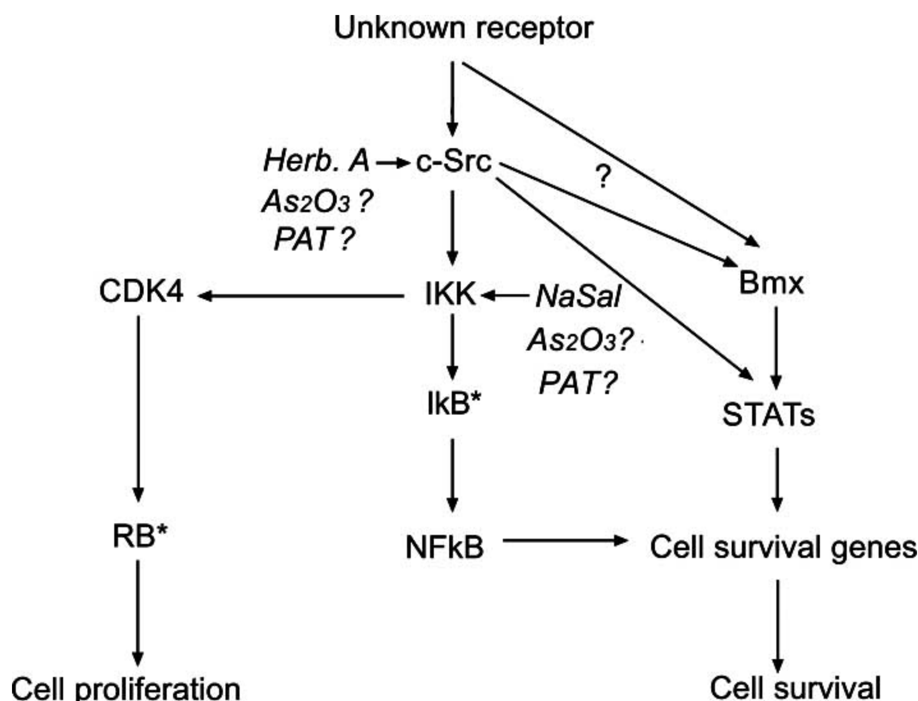

Fig. (1). Constitutively active signalling pathways in MF and SS cells. Inhibitors are given in italics. An asterisk indicates that this molecule has to be inactivated to get cell proliferation and NFkB stimulation. Rb = retinoblastoma protein, CDK = cyclin dependent kinase, NaSal = sodium Salicylate, Herb. A = Herbimycin A.

CELL DEATH INHIBITING (ANTI-APOPTOTIC) GENES ARE TARGETS OF NFKB AND STAT PROTEINS

The anti-apoptotic bcl-2 bcl-xL and mcl-1 genes have been reported to be regulated by NFkB and STAT proteins [40, 41]. Indeed the inhibition of NFkB and STAT factors by sodium salicylate and herbimycin A was paralleled with a reduced expression of these three genes [36]. The inhibition of bcl-2, bcl-xL and mcl-1 therefore seems to shift the equilibrium of the expression of pro- and anti-apoptotic genes to the side of the pro-apoptotic genes [39, 42]. No effect on the two agents on the expression on the pro-apoptotic bad and bax genes was detected.

INHIBITORS OF BCL-2 GENE EXPRESSION INHIBITORS ARE ALSO INHIBITORS OF NFkB AND STAT IN MF AND SS CELLS

It has been reported [43] that arsenic trioxide (As2O3) that is used against AML (acute myeloic leukemia) [44] is an inhibitor of bcl-2. Since MF and SS cells express bcl-2, As2O3 was tested for its ability to inhibit bcl-2 expression in these cells. Indeed non-toxic concentrations of As2O3 (1 – 2 uM) suppress bcl-2 expression and cause apoptosis of MF (MyLa 2059) and SS cells (HUT78, SeAx) in cell culture. The suppression of bcl-2 was paralleled by a repression of the DNA binding of NFkB and STAT protein. Thus the suppression of NFkB of STAT transcription factors may be the reason for bcl-2 repression. The expression of the anti-apoptotic bcl-xL and mcl-1 genes was also suppressed by the addition of As2O3, whereas the expression of the pro-apoptotic bax and bad genes remained nearly unchanged [45].

Since As2O3 is a known poison it may be not well accepted by patients. Since arsenic and antimony have very similar chemical properties, we looked for antimony compounds that have already been used for treatment of other diseases. Finally potassium antimonyl tartrate (PAT) was chosen, which has been used for the treatment against schistosoma and leishmania [46, 47] and has been reported to kill cells *in vitro* by oxidative stress [48].

Corresponding experiments as with As2O3 showed that PAT had the same effects as As2O3 [49]. It killed the MF and SS cell lines *in vitro*, suppressed the DNA binding of NFkB of STAT transcription factors and suppressed the expression of the bcl-2, bcl-xL and mcl-1. The expression of the pro-apoptotic bax and bad genes remained also unchanged. The effects occurred at concentrations of 5 -20 uM, depending on the cell line. These concentrations were 40 - 160 times lower than the LD50.

Both agents were tested for their effects in vivo. For this purpose immune deficient athymic nude mice were used, which were injected with MyLa 2059 cells. Tumors appeared after 2 – 6 weeks. The minimal concentrations to reach total remissions were 0.5 - 1 mM As2O3 and 1 - 2 mM PAT injected directly into the tumor. Since these high concentrations were applied locally, they had no toxic side effects. This result indicated hat MF tumor cells are much more resistant against anti-cancer agents, when they can grow as a tumor. This results explains why intravenous application of As2O3 in a 1 -10 uM range did not affect skin tumors of Sézary syndrome patients although the leukemic cells in the blood were killed [50].

THE EFFECTS OF As2O3 AND PAT ON OTHER CANCER CELL TYPES

As2O3 and PAT have also been tested for their efficiency against two other kinds of cancer. One is another kind of cutaneous T cell lymphoma, the anaplastic large cell lymphoma (ALCL) and the other is the squamous cell carcinoma of the skin. The tested ALCL cell lines (Karpas and Mac-1) were somewhat more sensitive to As2O3 and PAT (0.5 uM and 5 uM respectively) than the MF and SS cell lines (A. Tun Kyi and U. D. unpublished results).

The other type of cancer cells were squamous cell carcinoma cells. The sensitivity of SCC cell lines (HaCat, SCC-13, and A431) against As2O3 and PAT *in vitro* was the same as that reported for the MF and SS cell lines (U. D. unpublished data?). Malignant melanoma cells were as sensitive to As2O3 and PAT *in vitro* as MF and SS cells [51].

SUMMARY

The transcription profiling of Mycosis fungoides and the Sézary syndrome identified the NFkB and STAT factors and their signal transduction pathways as targets for new drugs for the treatment of these diseases. Prototypes of these drugs are the IKK beta inhibitor sodium salicylate and the src-type tyrosine kinase inhibitor herbimycin A. Both reagents inhibit the signalling through the NFkB and STAT pathways and cause cell death by the inhibition of cell survival genes.

As2O3 and PAT, which have been initially described as cell survival (anti-apoptotic) gene inhibitors had the same effects on NFkB and STAT activities and cell survival gene expression as sodium salicylate and herbimycin A. They may therefore also act on the same kinases as salicylate and herbimycin A. The effects of As2O3 and PAT could also be observed in a mouse model for MF (A. Tun Kyi, P. A. Oberholzer, and U. D. unpublished results).

Experiments in vitro have also shown that As2O3 and PAT can also other cancer cell types indicating that these two agents may also be effective against other types of cancer.

From the data from MF and SS one can conclude that the following strategy may be successful for the search of new anti-cancer agent:

1. Identify aberrant constitutive transcription factor activities in cancer cells.
2. Determine the involved signal transduction pathways and transcription factor targets.
3. Identify known inhibitors of these pathways and targets.
4. Test these inhibitors in vitro and *in vivo*.
5. Look for or develop derivatives of these inhibitors that are more effective and less toxic.

Transcription factor profiling is also a useful tool to investigate the pathogenesis of cancers.

It could be shown that the different NFkB and STAT transcription factors are activated at different stages of the diseases. This allows determining how the diseases have progressed and which treatment may the most suited one for the individual patient.

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