STAT 3 Activation in Head and Neck Squamous Cell Carcinomas is Controlled by the EGFR

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Abstract. Proliferation of squamous cell carcinoma of the head and neck (SCCHN) depends on epidermal growth factor receptor (EGFR) expression. As STAT 3 activation as well contributes to the cell growth in SCCHN, the interaction of STAT 3 and the EGFR is of great interest when considering treatment options through inhibition of STAT 3. We, therefore, evaluated the influence of blocking or activating the EGFR in human SCCHN cell lines and in vivo tumors on STAT 3 activation. We compared the effects on STAT 3 activation with the regulation of MAP Kinase under these conditions. We found that STAT 3 can be strongly inhibited via EGFR blocking in vitro as well as in vivo. However, the influence of EGFR regulation on the MAP Kinase pathway seemed to be very slight. These findings provide evidence that STAT 3 signal activity in head and neck carcinomas, which is partially responsible for proliferative activity, can be controlled via the EGFR.

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common malignant tumor worldwide. Whereas significant progress has been made in defining the molecular mechanisms of SCCHN progression, the overall survival of patients suffering from these tumors has not changed essentially in recent years. As SCCHN typically overexpress the epidermal growth factor receptor (EGFR), downstream signal transduction from the activated EGFR tyrosine kinase is thought to play a major role in proliferation of SCCHN cells.

The EGFR 3 family consists of four members: EGFR, ErbB2, ErbB3 and ErbB4 (1). The members of the EGFR family, particularly EGFR and ErbB2, are implicated in various forms of human cancers and serve as both prognostic markers and therapeutic targets. EGFR contains an extracellular ligand-binding domain, a single transmembrane region and an intracellular domain harboring intrinsic tyrosine kinase activity (2). Activation of the receptor tyrosine kinase requires ligand-induced dimerization that allows reciprocal transphosphorylation of residues within the catalytic domain leading to enzymatic activation and autophosphorylation of cytoplasmic tyrosine residues. It is known that this tyrosine kinase phosphorylation leads to activation of signal transducers via the Ras/ERK/MAPK pathway. Recent studies revealed, however, that EGFR activation may influence different signal transducers, for example the STAT proteins (signal transducers and activators of transcription). These were identified in the last decade as transcription factors that were critical in mediating virtually all cytokine-driven signalling (3-5). In addition to their central roles in normal cell signaling, recent studies have demonstrated that diverse oncoproteins can activate specific STATs (particularly STAT 3 and STAT 5) and that constitutively activated STAT signaling directly contributes to oncogenesis (6).

Furthermore, involvement of STAT 3 in chemoresistance of tumors has been described recently. The inactive cytoplasmic STATs are activated by phosphorylation, translocate to the nucleus and activate target gene transcription (7-9). Constitutive activation of STAT 3 has been detected in a wide variety of other cancers, including breast, prostate, renal cell, melanoma, ovarian, lung, leukemia, lymphoma, and multiple myeloma (10). Upon ligand-induced kinase activation and protein tyrosine phosphorylation, STAT 3 is recruited via its SH2 domain to tyrosine-phosphorylated motifs within receptor complexes and is itself phosphorylated on tyrosine 705 within its COOH terminus. Our understanding of the molecular details of its recruitment and activation by the EGFR are incomplete. Recently, it has been shown that STAT 3 directly binds to the cytoplasmic domain of the EGFR (11). It is not known whether STAT 3 activation or the MAPK cascade transfers the main signals for proliferation of SCCHN. Knowledge about these details in
signal transduction should lead to more therapeutical options in the treatment of SCCHN. By comparing the degree of STAT 3 and MAPK phosphorylation, we investigated EGFR signalling in EGFR blocked and activated cell lines, xenotransplants and in vivo human tumors.

Materials and Methods

To determine the signal transduction proteins STAT 3 and MAPK, we used tumor material from SCCN cell cultures (Detroit 562, C24, SCC 1624 and 10B), nude mice xenotransplants (Detroit 562) and human in vivo tumors. In vitro SCCHN cells were treated either with EGF or an EGFR-specific therapeutic antibody (see below). Nude mice bearing SCCHN tumors were treated intraperitoneally (i.p.) with the murine antibody Mab 425 as described below. Patients suffering from SCCHN were treated in a phase I protocol with the humanized EGFR antibody EMD 72000.

To elucidate the effects of EGFR activation and inhibition, the following formulae were used:

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\text{P}^* \text{ (sum of pixels)} = \frac{\text{EGF stimulated (P}^* \text{ whole protein) / P}^* \text{ phosphorylated protein}}{\text{untreated control (P}^* \text{whole protein) / P}^* \text{phosphorylated protein}}
\]

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\text{Activation index } a = \frac{\text{P}^* \text{ whole protein}}{\text{P}^* \text{ phosphorylated protein}}
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\text{Dephosphorylation index } d = \frac{\text{P}^* \text{ phosphorylated protein}}{\text{P}^* \text{ whole protein}}
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Antibody. The murine antibody EMD 55900 and its humanized version EMD 72000 were generously provided by E. Merck KGaA (Darmstadt, Germany). Monoclonal antibody EMD 55900 was induced by immunization of BALB/c mice with cells of the human carcinoma cell line A431. Phase I clinical trials revealed that the immune systems of patients receiving EMD 55900 produces HAMAs (human anti-mouse antibodies) against the drug because of the murine origin of these antibodies. For EMD 72000, mouse-complementary-determining regions have been grafted from EMD 55900 into a human IgG1 framework. The monoclonal antibodies
are highly specific for the human EGFR and recognize the deglycosylated region of the EGFR Mr -110,000 EGF-binding domain, but not the other peptides of the EGFR. Binding of both antibodies correlates with the number of EGF-binding sites and is strongest with A431 carcinoma cell membranes. Scatchard's analyses of 125I-labelled EMD 55900 binding to A431 membranes revealed the presence of two binding components: (a) a high-affinity, low-capacity component (Kd ' 10 pM, 5 3 108 sites/mg membrane protein); and (b) a relatively low-affinity, high-capacity component (Kd ' 1 nM, 2 3 1010 sites/mg membrane protein). Plasma-elimination half-lives of the murine (EMD 55900) and reshaped (EMD 72000) version were similar: short in the Cynomolgous monkey (26 h for EMD 55900 and 31 h for EMD 72000) and long in rats (240 h for EMD 55900 and 225 h for EMD 72000). Biodistribution studies of 125I-EMD 72000 in xenografted nude mice revealed a tumor:blood ratio of 1:2 on day 1 and 5:1 on day 18, respectively.

**Antibody-treated cell cultures.** Three different SCCHN cell lines were used in this investigation. Detroit 562 and SCC 1624 were obtained from ATCC (American Type Culture collection). UM-SCC 24 and UM-SCC 10B were kindly provided by Thomas Carey, University of Michigan, USA.

The cell lines were incubated with MAb 425 for 12, 24 and 72 h using three different doses (1, 10 and 100 µg / ml DMEM). After this, protein from the tumor cells was isolated directly for Western blot analysis.

**Tissue from antibody-treated xenotransplants.** For establishing cell lines as tumors on NMRI nu/nu mice (6-8 weeks of age), 2-3 x 10⁷
cells of each cell line in 200 μl MEM were inoculated s.c. into both flanks of NMRImnu mice (average body weight 25g). When the tumors reached a mean size of 1 x 0.5 cm, the monoclonal antibody EMD 55900 was applied i.p. After 12, 24 and 72 h, tumors were removed from the mice and directly frozen in liquid nitrogen and stored at -70°C.

Tissue from antibody-treated patients. In a phase I study, nine patients with stage III and IV SCCHN were treated with five administrations of the humanized anti-epidermal growth factor receptor monoclonal antibody EMD 72000 in three consecutive ascending dose groups. Loading doses of 100 mg (group I), 200 mg (group II) and 400 mg (group III) were followed by four weekly

Figure 3. Western blotting of phosphorylated and total MAPK protein in Detroit 562 tumor cells. The dosage of MAb 425 in cell culture assay was 1, 10 and 100 μg. The MAb 425 dose for treatment in animals was 2, 0.2 and 0.02 mg per injection. It is shown for Detroit 562 cells in vitro and in vivo (xenotransplantation) that MAPK phosphorylation is down-regulated dose-dependently after blocking the EGFR with MAb 425. Complete dephosphorylation of MAPK could not be observed.

Figure 4. The graph shows dephosphorylation of MAPK and STAT 3 after blocking the EGFR of cell lines Detroit 562, UM-SCC24, SCC 1624, UM-SCC10B as well as xenotransplanted and human in vivo tumors for 24 h in cell culture and 72 h in vivo. The total range of dephosphorylation indices is displayed. The dephosphorylation index is computed as described in the Methods section. It is demonstrated for each sample that blocking the EGFR led to a high degree of dephosphorylated STAT 3. Only partial MAPK dephosphorylation was observed in the case of Detroit 562 cells as well as UM-SCC10B cells. Human tumors as well as UM-SCC24 and SCC 1624 cells did not show dephosphorylation of MAP kinase.
EGFR blocking leads to dephosphorylation of STAT 3. Human in vivo tumors, xenotransplanted tumors and the four cell lines were treated with the monoclonal EGFR antibodies EMD 72000 (humanized MAb 425) and MAb 425. STAT 3 phosphorylation was down-regulated by treatment (see Figures 2 and 4). As seen in Figure 2, nearly complete dephosphorylation of STAT 3 could be observed in Detroit 562 cell culture samples as well as in Detroit 562 nude mice established tumors. Comparing human tumors treated during a phase I study with EMD 72000 with non-treated tumors of the same stage (UICC II-IV), EGFR blocking also led to a nearly complete down-regulation of the phosphorylated STAT 3. In all investigated tissues, a high degree of dephosphorylated STAT 3 could be detected (see Figure 4).

Dephosphorylation of MAPK is only partially regulated by the EGFR. As seen in Figure 3, MAP Kinase of Detroit 562 tumors was dose-dependently inactivated after EGFR blocking. However, computing the dephosphorylation index for all tumor samples revealed that only in Detroit 562 and UMSSC 10 B cells could MAP Kinase be inactivated after blocking the EGFR. Furthermore, the dephosphorylation levels were significantly below those observed in STAT 3 inactivation (see Figure 4). This could also be demonstrated in the case of the xenotransplanted tumors. The patient's tumors as well as the cell lines C24 and SCC 1624 did not show a significant inhibition of MAPK phosphorylation.

Discussion

In the present study, we describe the effects of EGFR activation or blockade on downstream signalling of STAT 3 and MAP Kinase in head and neck carcinomas. Activation of MAP Kinase and STAT 3 by stimulating carcinoma cells with EGF led to similar effects in all samples. However, blocking the EGFR only affected STAT 3 constitutively in all samples.

Increasing evidence supports the critical role of STAT 3 in tumor transformation and tumor progression. Extensive surveys in primary tumors and cell lines derived from tumors indicate that inappropriate activation of specific STATs occurs with surprisingly high frequency in a wide variety of human cancers (12). It has been shown that STAT 3 binds to the cytoplasmic domain of the EGFR (11). As well as STAT 3, MAP Kinase is known to react in consequence to EGFR activation. In our study, for the first time to our knowledge, tumor cell samples from anti-EGFR-treated patients suffering from head and neck cancers, nude mice transplanted human tumors and in vitro cell culture assays were integrated into one comparative investigation on signal transduction. The aim of this study was to find out whether the MAP Kinase-dependent pathway and/or STAT 3 was influenced equally by activation and blockade of the EGFR. This analysis should lead to evidence on how the cancer cell is able to affect downstream signalling by recruiting other intracellular mechanisms.

MAP Kinase is known to be activated via the signal transduction cascade of different receptor tyrosine kinases as well as integrins and ion channels. STAT 3 has been shown to be activated via IL-6 receptor signalling but also has been detected recently in the activated EGFR complex (11, 13), especially in the region between amino acid residues 1061 and 1123. As seen in Figure 4, dephosphorylation of STAT 3 after blocking the EGFR could be seen in each tissue sample. This suggests that no powerful mechanisms interfere with the STAT 3 activation at protein level. However, MAP Kinase activation seems to be strongly regulated via other pathways. Many modern anticancer drug discovery approaches have focused on targeting signal transduction pathways involving RTKs (e.g., ErbB2 and EGFR), farnesylated proteins (e.g., Ras), and nonreceptor cytosolic kinases (e.g., Raf, MEK, PI3k and Akt) (14). These important efforts resulted in several novel agents such as RTK monoclonal antibodies. Considering the results of the present study, the growth inhibitory effects on SCCHN of anti-EGFR therapy (15) may
therefore be mainly related to STAT 3 signal blockade. Otherwise cell growth regulation via MAP Kinases seems to be influenced to a minor degree via anti-EGFR treatment. We, therefore, conclude that, in case of sufficient growth inhibition of human tumors by anti-EGFR therapy, STAT 3 activation may play a major role in intracellular signalling and gene transcription. These results should lead to further investigations into identifying patients who would benefit from anti-EGFR therapy.

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References