Combination of sunitinib, cetuximab and irradiation in an orthotopic head and neck cancer model

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Background: Recent preclinical and clinical studies indicate beneficial effects from combining radiotherapy with either anti-angiogenic drugs or anti-epidermal growth factor receptor (EGFR)-targeting agent. To investigate the effect of combining these approaches, we evaluated in vivo the antitumor efficacy of the anti-angiogenic compound sunitinib, an oral, multi-targeted tyrosine kinase inhibitor that inhibits among others vascular endothelial growth factor (VEGF) receptors-1, -2 and -3, cetuximab, a mAb targeting the EGFR, and irradiation (RT) given alone and in combination.

Materials and methods: Investigations were carried out using a VEGF-secreting human head and neck tumor cell line, CAL33, with a high EGFR content, growing as orthotopic xenografts in nude mice. Three days after tumor cell injection, sunitinib (20 mg/kg, p.o.), cetuximab (1 mg/kg, i.p.), both 5 days/week seven doses, and RT (6 Gy, 3 days/week, four doses) were administered alone and in combination during 9 days.

Results: Concomitant administration of drugs produced a marked and significant supra-additive decrease, and the addition of RT completely abolished tumor growth. The drug association markedly reduced tumor cell proliferation (Ki67) and the number of the vessels, but enhanced cell differentiation.

Conclusion: The efficacy of this combination of sunitinib, cetuximab and RT may be of clinical importance in the management of head and neck cancer patients.

Key words: cetuximab, head and neck cancer, irradiation, sunitinib

introduction

In addition to direct effects on tumor cells, epidermal growth factor receptor (EGFR)-targeting drugs may also impart an indirect antitumor effect through anti-angiogenic activity. Thus, it has been reported that EGFR targeting in tumors may also modulate the migration of vascular endothelial cells and formation of tube-like structures from these cells [1]. More recently, we have shown the presence of a gefitinib-sensitive functional EGFR pathway in an immortalized microvascular endothelial cell line of human origin [2].

Several preclinical studies have examined the antitumor activity of inhibitors of EGFR and anti-angiogenic agents in combination and have demonstrated at least additive, if not synergistic, effects [3–5]. Previous experimental studies showed potential beneficial antitumor effects when combining anti-angiogenic agents with RT [6, 7], resulting in at least additive effects on tumor growth delay despite different radiation schedules, drugs and doses and combination regimens. Clinical research in this field is going on but additional preclinical studies are needed to further evaluate drug combinations, including the targeting of EGFR- and vascular endothelial growth factor (VEGF)-signaling pathways in association with RT.

We previously investigated the effects of combining anti-angiogenic treatment, EGFR targeting and irradiation (RT) on preclinical head and neck cancer models. We first evaluated an association between AZD2171, a highly potent, orally active, VEGF-signaling inhibitor; gefitinib, an EGFR tyrosine kinase inhibitor, and RT [8]. We then examined the antitumor effects of the clinically representative anti-angiogenic mAb bevacizumab combined with the anti-EGFR tyrosine kinase inhibitor erlotinib and RT [9]. In both studies, interesting supra-additive antitumor effects were observed. Since then a new treatment option for head and neck cancer management has appeared based on the RT–cetuximab combination for patients unlikely to tolerate a cisplatin-based chemoradiation treatment [10]. We took into consideration this clinically relevant RT–cetuximab tandem in order to reexamine the association with an anti-angiogenic agent since the benefits of anti-angiogenic therapy have recently been reported in head and neck cancer [11]. Sunitinib, which has now been tested on head and neck tumor patients with larger clinical trials ongoing [11], was chosen on account of its mechanism of action. The latter targets not only several players involved in angiogenesis.
such as endothelial cells and pericytes, but also tumoral cells themselves through the platelet-derived growth factor receptor [12], the presence of this receptor having been previously reported in human head and neck squamous cell carcinoma cell lines [13].

**materials and methods**

**chemicals**

Sunitinib was kindly provided by Pfizer (Groton, CT) and cetuximab by our institution’s pharmacy. Working solutions were prepared as follows: sunitinib (9.1 mg/ml) was diluted in citrate buffer (1 M, pH 3.5) and cetuximab (2 mg/l) diluted in 0.9% NaCl. For both drugs, the concentrations were adjusted so as to include the daily dose in 0.2 ml of drug suspension. Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin and glutamine were purchased from Whittaker (Verviers, Belgium). Fetal bovine serum (FBS) was obtained from Dutscher (Brumath, France).

**cell line**

CAL33, a cell line of human head and neck origin, was obtained from our institution (Centre Antoine-Lacassagne). This cell line exhibits high EGFR levels, (33,794 ± 624 fmol/mg protein high affinity sites determined by ligand binding assay) and produces VEGF (Cercina Onesto, CNRS-UMR6543, personal communication).

The cell line was maintained as monolayer culture in DMEM supplemented with 10% FBS by vol, 2 mM glutamic acid, 50 000 units/l penicillin and 80 μM streptomycin in a humidified incubator (Sanyo, Tokyo, Japan) at 37°C in an atmosphere containing 8% CO₂. Batches of 15 x 10⁶ cells were frozen in advance in FBS supplemented with 5% dimethyl sulfoxide by vol for injection into mice. Shortly before injection, cells were thawed and suspended in Ringer lactate.

**mice**

Six-week-old female Naval Medical Research Institute nude mice were purchased from Janvier laboratories (Le Genet-sur-Isle, France) and received, as i.m. orthotopic xenograft in the mouth floor as described by us [9], an injection of 0.5 x 10⁶ cells suspended in 200 μl of Ringer lactate (n = 10 animals per treatment condition). There were five animals per cage with food and water ad libitum; animals were killed by cervical disruption and tumors collected at day 12 after cell injection (a preliminary experiment demonstrated that, on the 12th day after tumor cell injection, tumor development in the mouth floor prevented correct feeding of the animals with consequent unethical suffering). As no signs of suffering appeared during the experiment (submissive attitude, weight loss, prostration, or vocalization) no animal had to be killed before the end of the experiment. Animal experiments were carried out in accordance with institutional guidelines: European Directive 86/609/EEC.

**treatment**

Beginning on the third day after cell injection, the mice were treated each week with relevant vehicle (controls), sunitinib (20 mg/kg, 5 days/week, 0.2 ml p.o., seven doses), cetuximab (1 mg/kg/day, 5 days/week, 0.2 ml i.p., seven doses) and RT (6 Gy, 3 days/week given every other day, 2 h after drugs, on tumor bed only, four doses) given alone or in combination (Figure 1). When coadministered, sunitinib and cetuximab were given simultaneously (given the relatively short duration of treatment (9 days) seven doses of each drug and four doses of RT were delivered). The doses of sunitinib and cetuximab were chosen in accordance with preliminary experiments (30 and 60 mg/kg p.o. for sunitinib and 1, 2 and 5 mg/kg/day, 5 days/week, 0.2 ml i.p. for cetuximab every day for 9 days), carried out on tumors from this same cell line xenografted in the flank of animals, so that each drug given alone had only partial effects on tumor growth.

**evaluation of drug effects**

On the 12th day after cell injection, animals were killed and weighed. Tumors were measured (tumor volume was determined by the formula \( TV = n/6 \times \text{length} \times \text{width}^2 \)) and weighed and bilateral radical neck dissection carried out (animals were killed by spinal cord dislocation and tumors were subsequently removed surgically and fixed in p-formaldehyde overnight). The effects of sunitinib, cetuximab and RT, alone or in combination, on tumor growth were evaluated by measuring, for all groups, the mean tumor volume (MTV). Combination ratios (CRs) as described previously by us [9] were calculated from the MTV of treated and untreated tumors at the end of the treatment period (day 12, after cell injection). This was done for control (MTV\(_{\text{control}}\)), treatment a (MTV\(_a\)), treatment b (MTV\(_b\)), treatment c (MTV\(_c\)), treatment a + b (MTV\(_{a+b}\)) and treatment a + b + c (MTV\(_{a+b+c}\)).

\[
\text{CR} a + b = \frac{\text{MTV}_a \times \text{MTV}_b}{\text{MTV}_{\text{control}}} \\
\text{CR} a + b + c = \frac{\text{MTV}_a \times \text{MTV}_b \times \text{MTV}_c}{\left( \text{MTV}_{\text{control}} \right)^2}
\]

If CR > 1, there were supra-additive effects and if CR < 1 infra-additive ones. Strictly additive effects were observed if CR = 1.

**analysis of tumor markers**

All analyses of tumor markers were carried out on the tumors which were collected 12 days after tumor cell injection. As no remaining tumor was observed in the triple combination, treatment effects on tumor markers could only be measured on single agents and on the sunitinib-cetuximab combination.

The expression of the micro-vessel marker VEGFR2 and of the proliferation marker Ki67 were evaluated using immunohistochemistry (Cell Signaling Technology rabbit mAb 55B11 and DAKO mAb, ref.: M7240 and MIB-1, respectively). The analysis of VEGFR2 concerned vessel loss and reduction in vessel area which were evaluated both on tumor center and tumor periphery. The analysis of Ki67 took into account the proportion of labeled cells. The proportion of well-differentiated tumor cells was determined by the classical hematoxylin–eosin–safron coloration.

Forty x10 magnification images of regions of high vascular density within the tumor were analyzed in order to quantify tumor angiogenesis. Whatever the studied marker (Ki67 or VEGFR2) was, the final value was the result of the examination of a maximum of 10, a minimum of two and a mean of five fields per tumor, which, due to the small volume of the tumor, allowed a thorough examination; a maximum of 10 and a minimum of zero (triple combination group) tumors were investigated by a pathologist for each treatment group.

<table>
<thead>
<tr>
<th>Cell injection</th>
<th>Tumor collection</th>
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<td>day 0</td>
<td>day 12</td>
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- Cetuximab
- Sunitinib
- Irradiation

Figure 1. Experimental schedule.
Comparison of tumor growth, node number, status of node invasion and the effects of treatment on VEGFR2, proportion of well-differentiated cells and Ki67 between different treatment groups were evaluated using the nonparametric analysis of variance (Kruskal–Wallis test) on GraphPad Instat software.

results

effects of cetuximab, sunitinib, RT and their combinations on tumor growth

Sunitinib, cetuximab and RT given alone significantly decreased tumor growth \(P < 0.05\), \(P < 0.001\) and \(P < 0.001\), respectively, as compared with the controls; Figure 2). The sunitinib–cetuximab combination produced a significant tumor mass decrease \(P < 0.001\) versus control; Figure 2). The effects of this combination were supra-additive (CR = 1.78). The triple combination cetuximab–sunitinib–RT gave the highest tumor growth inhibition (no detectable tumor in all treated animals without exception, \(P < 0.001\) versus controls; Figure 2) and gave supra-additive effects (CR > 2.4).

effects of cetuximab, sunitinib, RT and their combinations on lymph node invasion status

Given the small number of invaded nodes (<2) for each treatment group, no statistical differences could be
demonstrated between the different treated groups. Nevertheless, the invaded nodes/total nodes ratio for all treated groups (6/108) was significantly ($P = 0.028$) smaller than for the control group (5/24), thus indicating the global effect of treatments on node invasion status.

**effects of cetuximab, sunitinib, RT and their combinations on proliferation, tumor cell differentiation and tumor vessels**

Neither sunitinib nor RT alone had a significant impact on tumor cell proliferation ($P > 0.05$ versus controls; Figure 3) or differentiation ($P > 0.05$ versus controls; Figure 4). In contrast, cetuximab and the sunitinib–cetuximab combination significantly reduced cell proliferation ($P < 0.001$ and $P < 0.01$, respectively, versus controls; Figure 3) and enhanced tumor cell differentiation ($P < 0.001$ in both cases; Figure 4). Sunitinib, given alone or in combination with cetuximab, significantly decreased the number of vessels both at the center ($P < 0.01$ and $P < 0.05$ for sunitinib given alone and sunitinib + cetuximab, respectively; Figure 5A) and at the periphery ($P < 0.05$ for sunitinib given alone; Figure 5B) of the tumor. This was not the case for RT which seemed to increase (although not significantly) the number of vessels both at the center (Figure 5A) and the periphery (Figure 5B) of the tumor.

**discussion**

There are recent preclinical studies [14] and early clinical trials [15, 16] which indicate that targeting both EGFR pathway and tumor angiogenesis may be a useful strategy in the management of several tumor pathologies. Head and neck cancer is particularly relevant in this respect [17].

Interestingly, the combination of the two drugs presently investigated (sunitinib and cetuximab) produced supra-additive effects on the tumor mass with a CR value of 1.8. We recently made similar observations when applying on these same CAL33 cells, growing as a classical xenograft, the anti-angiogenic multi-target tyrosine kinase inhibitor AZD2171 associated with the anti-EGFR agent gefitinib [8] or with the association of the anti-VEGF antibody bevacizumab and the anti-EGFR agent erlotinib.
Interestingly, the supra-additive antitumor effects were found to be corroborated by an impact on cell differentiation. The tumors with the highest proportion of well-differentiated cells were those receiving the drug combination (Figure 4). One of the possible explanations for the presently observed supra-additive antitumor activity may lie in an optimal ‘vertical blockade’ of VEGF pathway not obtained by either agent alone, bearing also in mind that EGFR targeting leads to the inhibition of VEGF tumor secretion [18]. The effect of drugs on vessel count in tumors paralleled those observed in tumor cell differentiation, with the combinations leading to the lowest values both in the center of the tumor and its periphery (Figure 5A and B). As recently underlined by O’Reilly [19], there is a preclinical rationale to combine anti-angiogenic agents with radiation. The association of anti-EGFR therapy and radiation is based on consistent preclinical data confirmed at the clinical level, particularly in head and neck cancer [10]. In the present study, the sunitinib–cetuximab combination was considered as a whole when associated with RT. This triple combination was found to be particularly active since it completely eradicated tumor growth as no tumor could be detected at the end of the observation period.

In contrast to the drugs, RT induced tumor angiogenesis (Figure 5A and B). This phenomenon may be due, at least in part, to the postirradiation induction of EGFR signaling in irradiated cells which has been previously reported by others [20] and us [2]. It is known that VEGF secretion is under the control, at least in part, of EGFR signaling [21] which could explain the effect of RT on tumor angiogenesis observed in the present study.

In all, the present study provides another strong rationale for a combination of RT and EGFR pathway-targeting plus anti-angiogenic treatment [8, 14, 22]. The conclusion drawn from the specific experimental conditions with a head and neck cancer orthotopic xenograft model opens up the prospects of clinical applicability of the presently investigated treatment strategy, not only for the management of head and neck cancer but also in other major tumor pathologies such as non-small-cell lung cancer [23] and colorectal cancer [24] in which EGFR-targeting and anti-angiogenic treatments are particularly active.

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references