Microenvironment and Immunology

TLR3 as a Biomarker for the Therapeutic Efficacy of Double-stranded RNA in Breast Cancer

Bruno Salaun1, Laurence Zitvogel2,3,4, Carine Asselin-Paturel5, Yannis Morel2, Karine Chemin5, Clarisse Dubois5, Catherine Massacrier6, Rosa Conforti2,3, Marie Pierre Chenard6, Jean-Christophe Sabourin7, Aicha Goubar8, Serge Lebecque9, Michel Pierres10, Donata Rimoldi1, Pedro Romero1, and Fabrice Andre8

Abstract

The discovery of a targeted therapeutic compound along with its companion predictive biomarker is a major goal of clinical development for a personalized anticancer therapy to date. Here we present evidence of the predictive value of TLR3 expression by tumor cells for the efficacy of Poly (A:U) dsRNA in 194 breast cancer patients enrolled in a randomized clinical trial. Adjuvant treatment with double-stranded RNA (dsRNA) was associated with a significant decrease in the risk of metastatic relapse in TLR3 positive but not in TLR3-negative breast cancers. Moreover, we show the functional relevance of TLR3 expression by human tumor cells for the antitumor effects mediated by dsRNA in several preclinical mouse models carried out in immunocompromised animals. These 2 independent lines of evidence relied upon the generation of a novel tool, an anti-TLR3 antibody (40F9.6) validated for routine detection of TLR3 expression on paraffin-embedded tissues. Altogether, these data suggest that dsRNA mediates its therapeutic effect through TLR3 expressed on tumor cells, and could therefore represent an effective targeted treatment in patients with TLR3-positive cancers. Cancer Res; 71(5); 1–8. ©2011 AACR.

Introduction

The discovery of a candidate therapeutic target together with the identification of a highly sensitive population (predictive biomarker) is a major milestone in the development of new therapeutic agents in oncology. Several successes have been achieved recently, for instance with antibodies specifically inhibiting Her2/Neu/ERBB2 (1). Clinical benefit is attributable to the capacity of the drug to target a key oncogenic pathway aberrantly activated in tumor cells. Whether other molecules not specifically overexpressed by tumor cells could become drugable targets remained an open question. Here we show an example of this kind in which a drug [the double-stranded RNA (dsRNA) Poly (A:U)] can target its receptor TLR3 expressed on tumor cells leading to tumor regression in vivo.

Toll-like receptors (TLR) recognize pathogen-associated molecular patterns and are essential to generate potent antimicrobial proinflammatory immune responses. TLR3 is activated by viral or synthetic dsRNA (2). Such as Poly (A:U) sequences. Although initially described as specifically expressed by dendritic cells (3), TLR3 has recently been reported to be expressed by a broad array of epithelial cells including pulmonary cells (4) and hepatocytes (5). Interestingly, tumor cells such as breast cancers (6) or melanoma (7) can also express TLR3, which triggering may lead to apoptosis and/or to chemoattraction of cytotoxic lymphocytes to tumor beds (6–9). Six randomized trials carried out between 1970 and 1990 have evaluated the efficacy of dsRNA in oncology (10–15). Administration of dsRNA was associated with a significant clinical benefit in 2 trials (10, 11) and with a trend for a more favorable outcome in 3 other trials (12, 14, 15). This reproducible effect in small number of patients suggested that dsRNA could be highly effective in a subset of individuals that remained to be determined. The identification of TLR3 as one molecular target of dsRNA, together with the description of TLR3 expression by cancer cells, led us to hypothesize that TLR3 expressed by tumor cells could predict high sensitivity to dsRNA.

The current results show both the predictive value of immunoreactive TLR3 expression by tumor cells for the efficacy of dsRNA treatment in breast cancer patients, and the functional relevance of TLR3 expression by cancer cells for the antitumor effects mediated by dsRNA. Altogether, these
results establish a rationale for the assessment of TLR3 expression by tumors cells as a biomarker for the efficacy of dsRNA treatment in selected human cancers.

Materials and Methods

Generation of the 40F9.6 monoclonal antibody
BALB/c mice were immunized with a recombinant human His-tagged TLR3 extracellular domain recombinant protein (R&D systems). Mice received 1 primo-immunization with an emulsion of TLR3 protein and Complete Freund Adjuvant, intraperitoneally, a second immunization with an emulsion of TLR3 protein and Incomplete Freund Adjuvant, intraperitoneally, and 3 boosts with TLR3 protein, intravenously. Immune spleen cells were fused with X63.Ag8.653 immortalized B cells, and cultured in the presence of irradiated spleen cells. Hybrids were evaluated in a first screen for TLR3 binding using an ELISA developed for detection of binding to His-tagged recombinant TLR3 protein (R&D systems). Supernatants were screened in an immunohistochemistry screen for binding to 293T-TLR3 cells as a paraffin-embedded cell pellet, with no binding to 293T-TLR4 cells. Hybridomas selected from the initial screening were cloned by limit dilution techniques in 96-well plates, and subclones were tested in TLR3 ELISA and IHC for binding to 293T-TLR3 cells as a paraffin-embedded cell pellet.

Clinical trial and tumor samples
Primary tumors were obtained from 194 breast cancer patients included in a randomized trial. For trial inclusion, patients were selected to present operable T2/T3 breast cancer with involved axillary node, treated by mastectomy plus axillary dissection. Women were randomly assigned to receive either polyadenylic–polyuridylic acid (60 mg i.v. once a week for 6 weeks) plus postoperative locoregional irradiation with pelvic irradiation in premenopausal women or CMF (cyclophosphamide 100 mg/m² orally on days 1 and 8) for 6 monthly cycles. Overall 517 patients were included between 1982 and 1986 in this multicentric trial. Among these 517 patients, 197 were recruited at the Institut Gustave Roussy. Results of the trial have been reported previously (14, 16). TLR3 immunostaining (clone 40F9.6) was done on 194 paraffin-embedded tumor samples retrieved in Institut Gustave Roussy’s tumor bank.

TLR3 immunohistochemistry
After antigen retrieval in hot TRIS-EDTA-citrate buffer pH 7.8 and blocking of endogenous peroxidases with 3% H₂O₂, sections were stained with 40F9.6 antibody (10 μg/mL) for 1 hour. Staining was detected with Envision kit (DAKO) and Impact DAB Kit from Vector Laboratories. TLR3 staining was assessed by 2 independent investigators who were blinded to clinical characteristics and were not from similar institutions. TLR3 staining was considered positive if greater than 1% tumor cells were positive. TLR3 staining was done also on melanoma sections from patients of Centre Hospitalier Universitaire Vaudois according to local ethics protocols with patients’ informed consent. Melanoma tissue microarrays were purchased from US Biomax (TMA ME#802 and ME#241) and Tristar (TMA #69571049).

Mice, cell lines, and reagents
Animal experiments were carried out in agreement with local ethical guidelines. Rag2–/– common gamma chain –/– (Rag2 /– /– γc–/–) immunodeficient mice were obtained from Harlan and severe combined immunodeficiency-nonobese diabetic (SCID-NOD) immunodeficient mice were obtained from Charles River.
A 293T cell line (ATCC, #CRL-1573) was stably transfected with pUNO-hTLR3 plasmid or pUNO-hTLR4 plasmid (Invitrogen) and referred to respectively as 293T-TLR3 and 293T-TLR4. The human cell lines A375 (melanoma) and HCC1806 (breast adenocarcinoma) were obtained from the American Type Culture Collection, and stably transduced with lentiviruses expressing GFP, puromycin resistance gene, and shRNA specific for either control Lamin, (shLaminA/C, GAAGAGGGTGAACCTGATATCAAGAGATATCAGCTAC- CCTCTCTTC), hTRIF(shTRIF, AAGACCAAGCAGCCACTCCCAA- TCAAGAGGTTGAGTGGCGTCTGCTT) produced at Vectalys. Transduced cells were grown in the presence of puromycin, and tested for GFP expression to confirm the presence of the lentiviral vector. A375 cells were treated for 24 hours in vitro with 1000 U/mL of IFNγ2b (Introna, purchased from Schering Plough). Me 260 cells and lentiviruses expressing control siRNA (specific to Lamin A/C; siLamin) or siRNA specific to human TLR3 (siTLR3) were already described (7).
Poly (I:C) dsRNA was purchased from InvivoGen. The latter were regularly checked in agarose gels for integrity and were taken to prevent degradation due to shearing forces. Poly (A/U) dsRNA used in in vitro studies on human tumor cell lines and in vivo in SCID-NOD mice, was synthesized by Innate Pharma, and has been previously described (8).

Human tumor xenografts in immunodeficient mice
Human tumor cell suspensions freshly harvested from in vitro passaged tumor cell lines were injected s.c. in the flank (5.10⁶ cells per mouse for Me 260, 3.10⁵ for A375, and HCC1806). The treatment was started 1 week postgraftaftment, and consisted of either weekly i.v. injections with human IFNγ2b (10⁴ IU/mouse in 200 μL PBS) followed by Poly (I:C; 100 μg) 24 hours later for Me 260 cells, or daily injection of 200 μg (A375) or 500 μg (HCC1806) Poly (A/U), 5 times per week. Tumor growth was monitored weekly or biweekly with an electronic caliper, and expressed as volume (mm³).

Statistical analyses
Statistical analyses were done using the nonparametric, 2-tailed Mann–Whitney test. Both metastases and death, but not locoregional relapses, were considered as events. To evaluate dsRNA effect according to TLR3 expression in breast cancer patients, a multivariate Cox model was used. HR and 95% CIs are reported. Cox model was adjusted on prognostic variables (age and number of axillary nodes involved). Survival curves report the estimated probability of being free of...
metastases. These probabilities are adjusted for the clinical variables entered in the Cox model. Metastases-free survival rates reported in the text were calculated based on Kaplan–Meier method. Chi-square test was used to compare between categorical variables.

Results

Generation of a novel tool to detect TLR3 protein in paraffin-embedded tissues

To study TLR3 expression in human paraffin-embedded tissues, commercially available anti-TLR3 antibodies were tested, and none of them could specifically label paraffin-embedded pellets of 293T transfected with human TLR3 (293T-TLR3). We therefore generated new mAbs, including an IgG1 monoclonal antibody (mAB; clone 40F9.6) that specifically labeled 293T-TLR3 cells, but not control parental cells, nor 293T cells transfected with human TLR4 (Supplementary Fig. S1). This mAB strongly stained IFNα2b-treated A375 melanoma cells embedded in paraffin, whereas no detectable labeling could be observed of IFNα2b-treated A375 cells stably expressing a short hairpin RNA specific for human TLR3, further showing that 40F9.6 specifically recognizes human TLR3 expressed at physiological levels (Supplementary Fig. S1). Finally, 40F9.6 recognized recombinant human TLR3 extracellular domain in western blot experiments (data not shown).

TLR3 expression on tumor cells defines a subset of cancer patients highly sensitive to dsRNA

Immunohistochemistry with this mAB on tissue microarrays or paraffin-embedded tumor sections showed TLR3 expression by 50% of the melanoma tested (n = 99) and by 36% to 45% breast cancers in a cohort of 194 patients (Fig. 1A, Table 1, and Supplementary Table S1). Interestingly, this proportion of TLR3-expressing melanoma was similar to that observed in patient-derived melanoma cell lines by immunoprecipitation (7). We retrospectively assessed whether TLR3 expression could be a predictive marker of clinical response to systemic delivery of the TLR3 agonist Poly (A:U). We correlated TLR3 expression on primary breast tumors (determined by 2 independent investigators on immunostainings with 40F9.6) with the onset of metastatic relapse in a cohort of 194 breast cancer patients. These patients were included at the Institut Gustave Roussy in a multicentric randomized trial that compared Poly (A:U) treatment combined with radiation therapy to adjuvant chemotherapy (14; patients characteristics in Table 1). A TLR3-specific immunostaining

| Table 1. Characteristics of patients included in the clinical trial |
|-----------------|-----------------|-----------------|-----------------|
|                 | Investigator A   | Investigator B   |                 |
|                 | TLR3-negative breast cancer | TLR3-positive breast cancer | P |
|                 | n = 109 (64%)    | n = 81 (96%)    | 0.10           |
| Age (median, range) | 52 (28–65)      | 55 (30–66)      | 0.57           |
| Pathologic tumor size (median, range) | 30 mm | 30 mm | 0.99           |
| Lymph node status | 64 (59%)        | 35 (57%)        | 0.99           |
| Tumor grade | 45 (41%)        | 26 (43%)        | 0.99           |
| ER expression | 65 (61%)        | 36 (59%)        | 0.99           |
| Effect of dsRNA | 41 (39%)        | 25 (41%)        | 0.99           |
| Adjusted HR (95%CI)* | 1.01 (0.61–1.67) | 2.00 (1.03–3.89) | 0.91 (0.53–1.56) |
| 15 years TTM rate | 42% (27%–56%) | 56% (37%–71%) | 40% (26%–54%) |
| Chemotherapy arm | 33% (21%–46%) | 20% (8%–37%) | 37% (23%–52%) |

†HR for metastatic relapse, estimated from a Cox proportional hazard model adjusted on age (>50) and lymph node status (>3).

HR>1 favors dsRNA treatment.

Abbreviation: TTM, time-to-metastases.

NOTE: Characteristics according to TLR3 expression assessed by 2 independent investigators.

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Cancer Therapy with TLR3 Agonists

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was observed in 36% (investigator A) and 45% (investigator B) of breast cancers, without significant correlation with any clinical characteristics (except a higher rate of ER-positive cases in TLR3+ breast cancers for investigator B; Table 1). Scoring of TLR3 expression was discordant for 28 cases (16%). In a multivariate Cox analysis, adjuvant treatment with dsRNA was associated with a decrease in the risk of metastatic relapse in patients with TLR3-positive tumors \[HR = 2.00 (1.03–3.89) \text{ for investigator A, and 1.85 (1.04–3.32) for investigator B; see Table 1.}\] The estimated probabilities of being free of metastases according to TLR3 expression and adjuvant treatment are reported in Fig. 1B. When the analysis focused on patients with TLR3-positive tumor, the proportion of patients free of metastases at 15 years was 56% (95% CI, 37%-71%) in the Poly (A:U) arm and 20% (8%-37%) in the chemotherapy arm. Conversely, no significant clinical benefit could be observed after Poly (A:U) treatment in patients with TLR3-negative tumors, as the proportion of patients free of metastases at 15 years were 42% [27%–56%; Poly (A:U) arm] and 33% (21%-46%; chemotherapy arm). Similar results were obtained by the investigator B (Fig. 1B, right panels). When only patients treated with Poly (A:U) were taken into account, there was a trend toward an improved outcome in patients whose tumor overexpressed TLR3 as compared with those with...
TLR3-negative cancer [15-year metastasis-free survival: 56% (37–71%) vs. 42% (27–56%), \( P = 0.22 \)]. At the opposite, when the analyses focused on patients treated with adjuvant chemotherapy, a trend was observed toward a worse prognosis in patients who presented a TLR3-positive breast cancer as compared with TLR3-negative (\( P = 0.21 \) and 0.09 for investigators A and B respectively).

Altogether, these data, consistent between 2 different investigators, suggested that immunostaining of TLR3 on tumor cells could be a reliable approach to identify a subset of patients who are sensitive to dsRNA treatment. These data give rise to the hypothesis that dsRNA could mediate its antitumor effects through TLR3 expressed by cancer cells.

**TLR3 expression by tumors dictates the antitumor efficacy mediated by dsRNAs in vitro and in immunodeficient mice**

Based on these clinical data, we evaluated in vitro and in preclinical animal models whether the antitumor effect of dsRNA is mediated by TLR3 expressed by tumor cells. Both the melanoma cell line A375 and the breast cancer cell line HCC1806 underwent TRIF-dependent (i.e., TLR3 dependent) cell death after in vitro exposure to dsRNAs (Figs. 2A, 2C). Subcutaneous growth of both cell lines were severely impaired by intravenous injections of Poly (A:U) in NOD/SCID mice, which were treated as indicated. Tumor size is shown 28 days postengraftment. Each square represents an individual mouse (\( n = 8–17 \) for PBS group, \( n = 9–10 \) for Poly (A:U) group), and the dash is the mean. Data are pooled from 2 independent experiments.

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**Figure 2.** DsRNA induces inhibition of tumor growth in vitro and in vivo. Cell death (expressed as mean ± SEM) is reported in A375 melanoma cells (A) or HCC1806 breast cancer cells (C) stably expressing shTRIF or control shLamin and treated for 48 hours with 10 \( \mu \)g/mL Poly (I:C) or 1,000 \( \mu \)g/mL Poly (A:U). A375 (B) or HCC1806 (D) were injected s.c. in SCID-NOD mice, which were treated as indicated. Tumor size is shown 28 days postengraftment. Each square represents an individual mouse (\( n = 8–17 \) for PBS group, \( n = 9–10 \) for Poly (A:U) group), and the dash is the mean. Data are pooled from 2 independent experiments.
siTLR3 Me 260 tumors compared with controls (Fig. 3D). Altogether, these data support the finding that TLR3 expression by the tumor cell itself is mandatory for an optimal success of dsRNAs \textit{in vivo}, and suggest that dsRNA mediates its antitumor effects mainly via tumor-expressed TLR3.

**Discussion**

Several ligands for TLR3 are being developed in oncology including Hiltonol (Oncovir Inc.), Ampligen (Hemispherx), or PH3102 (Innate Pharma). As mentioned previously, earlier trials done in this field have emphasized the need for predictive biomarkers to identify highly sensitive population. In this study, we have reported that the subset of patients presenting a TLR3-overexpressing breast cancer derived high benefit from adjuvant dsRNA treatment. Importantly, the tool developed to quantify TLR3 expression (mAb clone 40F9.6) meets the criteria for a clinical use. Indeed, it specifically binds to TLR3, and the rate of discrepancies between readers is within acceptable range (18%) and comparable with what
is observed with estrogen receptor or Her2 (18). Several methodological points deserve further discussion. First, the clinical trial presented an imbalance in the use of radiation therapy across the 2 arms. To limit this bias, we have used incidence of metastases as study endpoint. Indeed, although radiation therapy dramatically improves locoregional control, there is no evidence that such modality of treatment could decrease incidence of metastases when compared with chemotherapy in patients treated with radical mastectomy (19). Second, although dsRNA improves outcome in TLR3-positive breast cancer, the prognosis was not significantly different between TLR3-positive and TLR3-negative breast cancer treated with dsRNA. The lack of 15-year survival difference between TLR3-positive and TLR3-negative patients treated with Poly (A:U) could be explained by an adverse prognostic value for TLR3 expression in patients not treated with the targeted agent. This phenomenon has been well described in the field of new targeted therapies. For instance, due to the adverse prognostic value of Her2-overexpression in untreated patients, patients with Her2-overexpressing breast cancer treated with trastuzumab have similar survival as the ones with Her2-negative breast cancers, although trastuzumab is providing dramatic outcome improvement in Her2-overexpressing breast cancer (20). Finally, although this study suggests that dsRNAs are mediating an antitumor activity via TLR3 expressed by cancer cells, we cannot exclude a role for the immune cells in TLR3-negative cancers. Indeed, the sample size is too small to exclude a small efficacy of dsRNA in TLR3-negative breast cancers.

Importantly, this clinical correlation was strongly supported by several xenograft models in immunodeficient mice showing a significant antitumor effect of TLR3 agonists that was totally independent of a fully functional immune system, but dependent on TLR3 expressed by tumor cells. Indeed, reduction of TLR3 expression by tumors using siRNA compromised dsRNAs efficacy, whereas its augmentation with human IFN\(\alpha\)2b, which acts only on human cells, enhanced it. Along these lines, we could show direct targeting of dsRNA to TLR3 expressed by tumor cells in vivo as human IL-6 could be detected in the serum of dsRNA-treated mice bearing TLR3 positive, but not TLR3 negative, tumors (Supplementary Fig. S2).

This study suggests that TLR3-mediated apoptosis could be the mechanism of the therapeutic effect mediated by dsRNA. Additional mechanisms of action could also contribute to the antitumor efficacy of such products in immunocompetent tumor-bearing individuals. First, TLR3 agonists are well-known immune adjuvants that promote antigen cross-presentation by dendritic cells and enhance antigen-specific CD8\(^+\) T cell responses (21–25). Moreover, we have previously shown in syngeneic mouse tumor models that Poly (A:U) can elicit the release of chemokines by TLR3-expressing tumor cells, and thereby modulate the tumor microenvironment and synergize with immunogenic chemotherapy and tumor vaccines (8). In addition, our unpublished data indicates that host IFN I receptor was required for an optimal therapeutic efficacy of Poly (A:U) against established TLR3-expressing tumors. Indeed, IFN I is needed for full DC maturation in response to dsRNA (26, 27) and it may also enhance the sensitivity of TLR3-expressing tumors to the drug (dsRNA).

In summary, this study strongly supports the clinical development of novel generations of TLR3-specific agonists. New prospective phase I/II clinical trials with these compounds should further validate TLR3 as a predictive biomarker for their therapeutic efficacy and document the potential additive role of IFN I receptor. These trials might be best implemented in cancer types that express high levels and frequency of TLR3, including advanced melanoma as shown here, but also in lung, cervical, or oesophagus cancers (our unpublished observations), renal cell carcinoma (28), hepatocellular carcinoma (29, 30), prostate cancer (31), or head and neck squamous cell carcinoma (32).

Disclosure of Potential Conflicts of Interest

C. Asselin-Paturel, Y. Morel, K. Chemin, C. Dubois, and C. Massacrier are employees of Innate Pharma, which is developing a TLR3 agonist in oncology.

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