Taxol Increases the Amount and T Cell–Activating Ability of Self-Immune Stimulatory Multimolecular Complexes Found in Ovarian Cancer Cells

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Abstract

It has been proposed that chemotherapy enhances tumor antigen (TA)–specific immunity. The molecular form of TA from ovarian tumor that activates cellular immunity is unknown. We report here identification of a novel molecular form of immunogenic TA for CD8+ cells named self-immune stimulatory multimolecular complexes (ISMMC). ISMMC consist of a molecular complex of polyosome/ribosome-bound ubiquitinated nascent HER-2 polypeptides. This complex is chaperoned by heat shock protein Gp96, which mediates ISMMC uptake by antigen-presenting cells through the scavenger receptor CD91. RNAs in ISMMC stimulate immature dendritic cells to secrete interleukin 12 and induce IFN-γ in peripheral blood mononuclear cells. ISMMC dissociate, retro-translocate from the lysosome to cytoplasm, and are processed to peptides by the proteasome. At subpharmacologic doses, Taxol increased the amount of ISMMC by three to four times and modified their composition by inducing the attachment of cochaperones of HSP70, such as the mitotic-phase phosphoprotein 11J. On a total protein basis, Taxol expanded more CD8+ cells, activated more phase phosphoprotein 11J. On a total protein basis, Taxol increased the amount of ISMMC by three to four times and modified their composition by inducing the attachment of cochaperones of HSP70, such as the mitotic-phase phosphoprotein 11J. On a total protein basis, Taxol expanded more CD8+ cells, activated more

Introduction

Chemotherapy by Taxol in advanced ovarian cancer results in recovery of CD8+ T-cell function (1–3). Potentiation of immune response has been attributed to Taxol-induced tumor apoptosis, which releases tumor antigens (TA). However, the immunogenic form of TA in cancer cells and how Taxol increases immunogenicity are unknown.

We hypothesized that to activate immunity to cancer cells, TA should be linked to molecules that mediate its uptake by antigen-presenting cells (APC) and to activators of proinflammatory cytokines in APC. We also hypothesized that for self-immunization, the TA, as a complete molecule or as a shorter precursor of the CTL-activating peptide (pCE), should be linked to ubiquitin in positions that are sensitive to deubiquitinating enzymes. Binding the optimal numbers of ubiquitin molecules in the correct position ensures pCE-TA binding to the proteasome (4). The ubiquitin-linked form of pCE is essential for generating immunogenic peptides because one molecule of pCE-TA competes with distinct ubiquitin-linked molecules for proteasomal degradation.

Thus, we hypothesized that TA form molecular complexes with accessory molecules implicated in these processes. We named these unknown complexes self-immune stimulatory multimolecular complexes (ISMMC). To identify the composition and function of ISMMC, we investigated which of the subcellular components of SK-OV-3 cells activated TA-specific lytic CD8+ cells in the absence of exogenous costimulation signals or the proinflammatory cytokine interleukin 12 (IL-12).

To characterize the activation of CD8+ cells, we quantified expansion of cells bearing T-cell receptor density (TCR) for the peptide HER-2 (369–377, also designated E75) in complex with the human leukocyte antigen HLA-A2 (5, 6). We found that the heavy ribosomal fraction, i.e., polysomes separated in sucrose gradient, was the most effective ISMMC with regard to E75-TCR+ cell proliferation and differentiation. The minimal ISMMC for E75-TCR+ cells consisted of a pentamolecular complex formed by nascent HER-2 polypeptides, ubiquitin, mRNA, rRNA, and Gp96.

Treatment of SK-OV-3 with Taxol significantly increased (by four times) the amount of immunogenic polysomal ISMMC per cell and its resistance to vacuolar proteases. The larger amount of immunogenic Taxol-induced ISMMC can activate more T cells than the native ISMMC from untreated tumors.

Materials and Methods

Cells lines. The cell lines SKOV-3, SKOV-3A2, and 2774 were cultured as described (7). Peripheral blood mononuclear cells (PBMC) from five distinct donors, designated PBMC-1 to PBMC-5, were isolated from distinct healthy HLA-A2+ donors. Immature dendritic cells (iDC) used as APC were generated as described (8). SK-OV-3 cells were cultured with GM-cycillin (Boehringer) for 2 weeks before expansion. Mycoplasma and bacteria contamination tests were negative. LPS in ribosome nascent chains (BNC) was determined with the Limulus amoebocyte lysate assay. RNCs were essentially free of LPS. The amount of LPS determined (pg/mL) was below of that needed to activate IL-12 in GM-CSF + IL-4–cultured iDC (1–10 ng/mL) and mature APC (20 ng/mL; ref. 9). LPS was in the same range with that reported for RNA preparations that were considered RNA

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Results

SKOV-3 treated with subpharmacologic concentrations of Taxol activates CD8+ cells. To identify what concentration of Taxol induces tumor immunogenicity, we determined its effects on tumor cells and leukocytes. Twelve hours after treatment with 42 nmol/L Taxol (equivalent to the pharmacologic dose in humans), both CD8+ and CD83+ dendritic cells (DC) decreased in number by 90% and the expressions of CD83 and HLA-DR molecules on surviving cells decreased by 80%. A dose of Taxol 10 times less than the pharmacologic dose in humans (i.e., 4.2 nmol/L) only marginally affected (9% apoptotic cells) the viability of iDC, PMBC, and nonadherent PMBC. Taxol induced accumulation of most tumor cells in the G2-M phase when used at 42 nmol/L but increased the number of SK-OV-3 in the S phase when used at 0.42 and 4.2 nmol/L (Supplementary Table S1A). These findings are supported by reports that subpharmacologic, but not pharmacologic, doses of Taxol enhance the functional levels of the eukaryotic initiation factor eIF-4E and activate translation in tumor cells before inducing apoptosis (12, 13).

To determine whether Taxol-treated SK-OV-3 contain ISMMC, Donor-1 iDC were added to untreated and Taxol-treated live SK-OV-3. Nonadherent PMBC (>95% T cells) from healthy HLA-A2+ Donor 1 (Donor-1 PMBC) were added 1 h later. We found a significant increase (>2-fold) in the number of CD8+ E75–high TCR (TCRhi), high-Perforin cells activated by 4.2 nmol/L Taxol-treated SK-OV-3 relative to cells activated with untreated SK-OV-3 (Supplementary Table S1B).

Differentiation of E75-TCR+ cells to Perforin+ cells requires both peptide and IL-2 as cofactors and is regulated by IFN-γ (14). We tested the effects of IL-12, E75 peptide plus IL-12, and polysomal fraction IV [also called ribosome nascent chain IV (RNC-IV)] from untreated SK-OV-3 on the differentiation of E75-TCR+ cells. IL-12 activated 6.5 times more E75-TCRhi Perforin+ cells from the existent precursors and 2.7 times more E75-TCRhi Perforin+ cells than did the untreated and Taxol (4.2 nmol/L)–treated SK-OV-3, respectively (Supplementary Table S1C).

RNC-IV of untreated SK-OV-3 contained both APC and T-cell stimulatory components because it activated thrice more E75-TCRhi Perforin+ cells than did IL-12 alone (Supplementary Table S1C). The positive control, peptide E75 + IL-12, activated 3.6 times more E75-TCRhi Perforin+ cells than IL-12 did. Overall, RNC-IV activated 19.5 times more (6.5 × 3) E75-TCRhi Perforin+ cells than SK-OV-3 and 8.1 times more E75-TCRhi Perforin+ cells than Taxol-treated SK-OV-3 did. These findings are compatible with the hypothesis that ISMMC are present in SK-OV-3 and that Taxol increased their amount.

Ribosomes from Taxol-treated SK-OV-3 are more effective activators of cytolyis than cytoplasmic and nuclear components. To verify the hypothesis that ISMMC are formed by tumor polysomes, equal amounts of protein of the subcellular fractions from Taxol (4.2 nmol/L)–treated SK-OV-3 were used to stimulate PMBC-1 in the absence of exogenous IL-12. PMBC-1 activated by the ribosomal fraction showed greater lysis of SK-OV-3.A2 than PBMC activated by the nuclear and cytoplasmic membrane fractions (Fig. 1A). SK-OV-3 lysis by ribosome-activated PMBC-1 was comparable with that mediated by RNC-IV–activated PMBC-1 (Fig. 1A). To verify that cells were activated by ISMMC, PMBC were stimulated with polysomal fractions RIBO-17 and RIBO-17-Tx (defined in Fig. 2A–C). Inhibition of SK-OV-3 lysis by specific antibodies to lymphocyte antigens indicated that CD8+ and CD56+ cells, but not CD4+ cells,
cells, were activated by ISMMC from both Taxol-treated and untreated SK-OV-3. Anti-CD4 had a small and insignificant inhibitory effect (Fig. 1B).

Ribosomes from Taxol-treated SK-OV-3 induced IL-12 and IFN-γ in PBMC. Activation/maturation stimuli delivered to APC determine whether APC functions as an inducer of Th1 and Th2 or induces immune tolerance (7, 15, 16). Differentiation of CD8+ cells to effectors requires proinflammatory cytokines, such as IL-12. To identify which fraction of SK-OV-3 induced IL-12, PBMC-1 were activated with the cytoplasmic plus membrane, nuclear, and ribosomal fractions, and the amount of IL-12 (p70) produced was determined. The nuclear and ribosomal, but not the cytoplasmic plus membrane, fractions from both untreated and Taxol-treated SK-OV-3 induced IL-12 (Fig. 1C). At equal RNA concentrations, ribosomes from Taxol-treated SK-OV-3 induced more IL-12 than ribosomes from untreated cells by almost 50%. To confirm that RNA induced IL-12 in these APC, we used as the positive control the RNC-KIF fraction from SK-OV-3 as the IL-12 inducer. RNC-KIF consists of isolated polysomes from SK-OV-3 infected with the recombinant influenza strain A/PR8/34 (see Materials and Methods). Influenza virus RNA is a potent inducer of IL-12. RNC-KIF induced significantly (thrice) more IL-12 than SK-OV-3 ribosomes, confirming that polysome-bound RNA induced IL-12.

To identify the pathways of activation of IL-12 by ISMMC, we investigated the effects of specific inhibitors. IL-12 is produced by signaling from transmembrane nucleotide receptors of the P2Y family in response to poly-(A) tail and/or by intracellular RNA receptors in response to the RNA sequence. Suramin, bafilomycin A, and chloroquine were added at the beginning of incubation with agonists at concentrations of 30, 50, and 25 mmol/L, respectively. The percentage inhibition in cytokine production by chloroquine, bafilomycin, and suramin is calculated in relation to the amount of cytokine found in cultures not treated with inhibitor. IL-12 induced by RNC-IV, RNC-KIF, R-Tx, R, N-Tx, and N versus 0.0 (unstimulated), C+M and C+M-Tx; P < 0.01. Inhibition of IL-12 and IFN-γ by chloroquine; P < 0.02. ND, not done.

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not involved in IL-12 production. Bafilomycin A and chloroquine inhibited the amount of IL-12 induced by RNC-IV and the positive control activator RNC-KIF by 45% and 85%, respectively (results not shown).

Chloroquine disrupts endocytic vesicles, which release internalized proteins and nucleic acids into the cytoplasm. In contrast, bafilomycin A inhibits vacuolar ATPases but does not induce release of the vacuole contents into the cytoplasm (11). Because chloroquine mediated almost complete inhibition of cytokines, whereas bafilomycin A did so only partially, exogenous RNCs likely reached the vacuolar compartments (i.e., endosomes and/or lysosomes) and activated Toll-like receptors (TLR-3 and TLR-8) in situ. Part of the RNA in ISMMC translocated to the endoplasmic reticulum (ER) and activated TLR-8 and/or RNA exited the vacuoles and activated cytoplasmic RNA receptors. Similar results were obtained for inhibition of IFN-γ induction by chloroquine in the same experiment (Fig. 1D). Therefore, induction of IL-12 required internalization of ISMMC and activation of intracellular RNA receptors.

Subpharmacologic doses of Taxol increase the amount of heavy polysomes in live SK-OV-3. To identify how Taxol changes ISMMC, we quantified the amount of RNA in the polysomes of SK-OV-3 separated by sucrose-gradient centrifugation. Compared with untreated cells, 4.2 mmol/L Taxol increased by four times the amount of RNA and proteins attached to polysomes (Fig. 2A and Supplementary Table S2) without shifting the ribosome peaks. These changes are consistent with activation of RNA synthesis by low concentrations of Taxol followed by ribosome aggregation due to "stalling" translation (17).

We characterized distribution of the nascent HER-2 polypeptides on ribosomes. HER-2 polypeptides attached to RNA in the sucrose-gradient (RIBO) fractions from untreated SK-OV-3 (RIBO-13, RIBO-16, and RIBO-17) and from Taxol-treated SK-OV-3 (RIBO-13-Tx, RIBO-16-Tx, and RIBO-17-Tx) were identified by probing with mAb-19. The recognition site of the HER-2–specific mAb-19 is the RIBO-16-Tx, and RIBO-17-Tx contained more ubiquitin (Fig. 2A). Part of the RNA in ISMMC translocated to the endoplasmic reticulum (ER) and activated TLR-8 and/or RNA exited the vacuoles and activated cytoplasmic RNA receptors. Similar results were obtained for inhibition of IFN-γ induction by chloroquine in the same experiment (Fig. 1D). Therefore, induction of IL-12 required internalization of ISMMC and activation of intracellular RNA receptors.

Oligoubiquitinated HER-2 polypeptides are present on polysomes from SK-OV-3. Taxol increased the amount of ubiquitin-conjugated nascent polypeptides and brought Mpp-11J to ISMMC. The proteasome preferentially degrades ubiquitin–linked proteins to peptides. At least four ubiquitin molecules linked to a protein are needed for its conjugation to proteasome. Proteins linked with one to two ubiquitin molecules, i.e., oligoubiquitin–ubiquitin(1–2) linked, are preferentially linked with three to five additional ubiquitin molecules after endocytosis, translocated to cytoplasm, and degraded by the proteasome. The proteasome degrades ubiquitin(2–6)–linked proteins with priority over the ubiquitin(1–6)–linked proteins (18, 19).

We reprobed the blots with P4D1, an antibody that recognizes both oligo{ubiquitin(1–6)–linked and poly{ubiquitin(1–6)–linked proteins. More of an ubiquitin–linked polypeptide of the same length as p53HER-2 was present in RIBO-16-Tx and RIBO-17-Tx fractions than in RIBO-16 and RIBO-17. These results suggested that p53HER-2 consisted of an ubiquitin–linked HER-2 fragment. The amount of ubiquitin–linked p53 was several times higher in Taxol-treated SK-OV-3 than in untreated SK-OV-3. RIBO-16-Tx and RIBO-17-Tx contained more ubiquitin(1–6)–linked proteins than RIBO-16 and RIBO-17 did. Ubiquitin–linked chains of the same length as p91HER-2 were not detected in RIBO-16 and RIBO-17, suggesting that p91HER-2 was not ubiquitin–linked (Fig. 2C).

Gp96 was present in RIBO-16-Tx and RIBO-17-Tx in four times the amount present in RIBO-16 and RIBO-17 (Fig. 2D and Supplementary Fig. S1B and C for fraction RIBO-17-Tx after purification in a longer sucrose gradient). Mpp-11J, a cochaperone that binds

Figure 2. Taxol increases the amount of polysomal RNA–protein complexes in SK-OV-3. A, sucrose-gradient separation profile of ribosomes from control SK-OV-3 (Δ) and from SK-OV-3 treated with 4.2 mmol/L Taxol (○) or 42 mmol/L Taxol (●). The increases in total protein and RNA in polysomes by Taxol are shown in Supplementary Table S1A and B. B, sucrose-gradient ribosomal (R) fractions 16 and 17 from control and 4.2 mmol/L Taxol treated (16T and 17T) SK-OV-3 contain two main HER-2 fragments of 53 and 91 kDa (1* and 3*, respectively). The amount of each HER-2 polypeptide, detected with mAb-19, was quantified by scanning densitometry. C, Taxol increases the amounts of poly{ubiquitin(1–6)–linked proteins in RIBO-16-Tx and RIBO-17-Tx compared with RIBO-16 and RIBO-17. Ubiquitin(1–6)–linked polypeptides of similar length as the p53HER-2 fragment are present. Similarly, high amounts of poly{ubiquitin(1–6)–linked proteins are present in RIBO-13-Tx (Supplementary Fig. S1A). The ubiquitin–specific mAb P4D1 was used for detection. Taxol induced association of higher amounts of Gp96 (Δ) and the ribosomal cochaperone Mpp-11J (●) with RIBO-16-Tx and RIBO-17-Tx than with RIBO-16 and RIBO-17.
polypeptides exiting the ribosomal tunnel and recruits the multifunctional Hsc-70 (20), was present in RIBO-16-Tx and RIBO-17-Tx but was undetectable in RIBO-16 and RIBO-17 (Fig. 2E). Therefore, treatment with Taxol increased the amount of large polysomal complexes by linking together more ribosomes, ubiquitin(n)-linked proteins, Gp96, and Mpp-11.J. The amount of RNA in the RIBO-17-Tx fraction quadrupled and the amount of bound proteins more than tripled compared with the RIBO-17 fraction of equivalent size from untreated SK-OV-3.

RIBO-17-Tx expanded more E75-TCR+ CD8+ cells than RIBO-17 did. To characterize the activation of CD8+ cells by ISMCC, PBMC were stimulated with (a) RIBO-17-Tx compared with RIBO-17 are indicated by ×1.56 and ×1.85. B. RNaseA-treated RNC-III is a more efficient activator of E75-TCR+ cells than non-RNaseA-treated RNC-III. Donor-3 PBMC were stimulated with 1,000 ng of total RNA from non–RNaseA-treated RNC-III (Δ), RNC-III treated with RNaseA (■), non–RNaseA-treated control 2774 cell polysomes (○), control 2774 cell polysomes treated with RNaseA (●), 5 μg/mL free peptide E75 (♦), or remained unactivated (□). "Control" indicates that polysomes were from 2,774 cells, which express low levels of HER-2. Seven days after activation, E75-TCR+ cells were present in all cultures. With the exception of E75-TCR+ cells activated by RNaseA-treated polysomes, most E75-TCR+ cells decayed on day 16. E75-TCR+ cell numbers activated by RNaseA-treated RNC-III versus E75-TCR+ cells activated by non–RNaseA-treated RNC-III (P < 0.05, >2.5 times increase). C. SKOV-3 polysomes (Polysomes) are more efficient activators of CTL, which lysed the HLA-A2–matched SKOV-3.A2 cells than control, unstimulated CTL plus IL-12, and CTL activated by free peptide E75 (at 5 μg/mL) plus IL-12 (E75). Columns, results of a 4-h CTL 51Cr-release assay (% specific lysis by polysomes and E75-activated CTL versus NP; P < 0.05, >2.5 times increase) are from one of two representative experiments done in triplicate. D. SKOV-3 polysomes (▲) are more effective activators of CTL than isolated mRNA from the same SK-OV-3 (■). Nonadherent Donor-3 PBMC were activated with autologous iDC pulsed with equal amounts (1,000 ng) of RNA from polysomes and isolated mRNA from SK-OV-3. Equal numbers of CD8+ cells activated by polysomes and mRNA were used as effectors in a 4-h CTL assay (% specific lysis; polysome-activated versus mRNA-activated; P < 0.03, more than thrice the increase). Points, results from one experiment done in triplicate.
RIBO-13 and RIBO-13-Tx were much weaker activators of E75-TCR⁺ cells (data not shown). Because Taxol increased the amount of protein and RNA in ISMMC by a factor of 4, Taxol-induced ISMMC can differentiate at least six times (1.5 × 4) more antigen-specific cells than native ISMMC can and expand eight times more Perforin⁻ antigen-specific CD8⁺ cells than native ISMMC can.

Ribosomes generated by RNaseA digestion expanded more E75-TCR⁺ cells than polysomes did. To identify how ISMMC activate CD8⁺ cells, we treated the large peak (RIBO-13 to RIBO-18) into two peaks by longer (6 h) sucrose-gradient centrifugation (Supplementary Fig. S2A). The corresponding ISMMC were designated RNC-I, RNC-II, RNC-III, and RNC-IV. RNC-III included RIBO-13, and RNC-IV included RIBO-17.

To determine whether mRNA-linked polysomes were sufficient for activating CD8⁺ cells, we treated RNC-III with RNaseA. The specificity of mammalian RNaseA is close to the that of human RNaseA. RNaseA preferentially digests single-stranded RNA (i.e., mRNA and tRNA) but has modest activity for double-stranded (i.e., ribosomal) RNA. Treating RNC-III with RNaseA enhanced its ability to expand Donor-3 CD8⁺ cells by a factor of 5.4 compared with RNC-III untreated with RNaseA (Fig. 3B, day 16).

RNaseA-treated RNC-III expanded four times more E75-TCR⁺ cells than the free peptide E75 + IL-12 did. RNC-III polysomes untreated with RNaseA only transiently expanded E75-TCR⁺ cells (Fig. 3B, day 7).

To confirm that CD8⁺ cells activated by polysomes expanded more effectors than peptide E75 did, we measured lysis of SK-OV-3.A2 by PBMC-3 activated by peptide E75 and polysomes (Fig. 3C). At the same number of CD8⁺ cell effectors, lysis by polysome-activated effectors was 60% higher than by peptide E75 + IL-12-activated effectors. Five micrograms of E75 corresponded to 900 μg of complete HER-2 protein. The amount of total proteins, including HER-2 bound to 1 μg RNA, did not exceed 4 μg. Assuming that 10% of cHER-2 is from total polysomal bound protein, then polysomes were 20 times more effective than free peptide E75 in activating CD8⁺ cells.

To determine whether mRNA could replace ISMMC for activation of CD8⁺ cells, PBMC-3 were activated by polysomes and mRNA isolated from the same SK-OV-3. ISMMC were 2.5 times more effective in activating tumor cell lytic CD8⁺ cells than purified mRNA was (Fig. 3D). Therefore, polysomes were more effective activators of cytolytic CD8⁺ cells than mRNA was.

The most efficient activators of E75-TCR⁺ IFN-γ⁺ cells are present in RNC-IV. Native and Taxol-induced ISMMC were more effective than peptide E75 in expanding lytic CD8⁺ cells. This finding raised the hypothesis that the ISMMC-linked pCE is in a form that favors its processing and E75 presentation. Taxol-induced ISMMC were only slightly better activators of perforin than native ISMMC. Expression of IFN-γ precedes expression of perforin in CD8⁺ cells. TCR-induced death of IFN-γ⁺ cells also eliminates CD8⁺ cells expressing IFN-γ and perforin (14). To identify the pCE of E75, we analyzed the possible relationship between the cHER-2 and HER-2 polypeptides and the immunogenicity of native ISMMC.

The amount of cHER-2 decreased from RNC-I to RNC-IV, indicating, as expected, that less cHER-2 was synthesized on heavy...
than on light polysomes (not shown). The amount of ubiquitin\(n\)\()\)-linked proteins peaked in the ribosomal fraction 8, which was part of RNC-II, and decreased in RNC-III and RNC-IV (Fig 4A).

We primed PBMC-3 with the proteins bound to equal amounts of RNA (as a constant APC activator) from all four ISMMC: RNC-I, RNC-II, RNC-III, and RNC-IV. RNC-III contained the most efficient pCE for activation and proliferation of CD8+ cells specific for multiple ribosomal-bound TA. CD8+ cells activated by RNC-III expanded by more than thrice compared with CD8+ cells activated by RNC-I (Fig 4B). At equal amounts of total RNA, the total amount of proteins in RNC-III was 2.7, 1.4, and 1.3 times lower than that in RNC-I, RNC-II, and RNC-IV, respectively (Supplementary Table S2).

We then quantified E75-TCR+ cells expanded by the four ISMMC listed above. To increase the sensitivity of detection, we measured cells expressing IFN-\(\gamma\). There were 4.0 and 5.5 times more E75-TCR+ CD8+ IFN-\(\gamma\)-cells in RNC-II-activated and RNC-IV-activated PBMC-3 than in non–RNC-activated and RNC-I-activated PBMC-3 (Fig 4C). Because twice less RNC-II and RNC-IV proteins than RNC-I proteins were used for activation, we concluded that RNC-IV was the most efficient immunogen for E75-TCR+ CD8+ IFN-\(\gamma\)-cells.

We noted that RNC-III contained six HER-2 fragments longer than p91HER-2. Ubiquitin-linked P4D1-reactive proteins of the same length were not detected. RNC-III and RNC-IV contained similar amounts of p91HER-2 and p53HER-2. Both RNC-III and RNC-IV contained ubiquitin\(n\)-linked proteins (Fig 4D, top of the gel). p35HER-2 was oligoubiquitinated, but p91HER-2 was not ubiquitin linked.

To contain both E75 (amino acids 369–377) and the mAb-19-recognized epitope (amino acids 377–394), p35HER-2 should consist of a 45-kDa HER-2 polypeptide linked to one, but not two, ubiquitin monomer. If we subtract the ubiquitin mass (8,000 Da) from the total mass of p35HER-2 (53,000 Da), the result is a 45,000-Da HER-2 fragment. If we divide the mass of this fragment by the total mass of p53HER-2 (53,000 Da), the result is a 45-kDa HER-2 polypeptide linked to one, but not two, ubiquitin molecules. Therefore, each p53HER-2 molecule is likely to contain one ubiquitin, but not two.

Because E75 constitutes 2.2% and 1.1% of p53HER-2/p45HER-2 and p91HER-2, respectively, our results suggested an additional source of E75 precursor, likely in ubiquitin\(n\)\()\)-HER-2. However, mAb-19 cannot distinguish between ubiquitin\(n\)\()\)-p91 and ubiquitin\(n\)\()\)-p35HER-2. To identify polyubiquitinated HER-2 peptides, we treated RNC-III and RNC-IV with the deubiquitinating enzyme Iso-T. Iso-T cleaves multiubiquitin and branched ubiquitin chains, leaving ubiquitin monomers intact (9). After digestion with Iso-T, the ratio p53HER-2 (RNC-IV/RNC-III) and p91HER-2 (RNC-IV/RNC-III) increased similarly (by 1.36 and 1.54 times, respectively), indicating that only part of both p53HER-2 and p91HER-2 was polyubiquitinated. However, the overall increase of p53HER-2 in RNC-IV compared with RNC-III was 2.48 times, whereas that of p91HER-2 was only 1.6 times.

Three longer HER-2 polypeptides were detected by mAb-19 after Iso-T digestion (Fig 5C). p105HER-2 was present in equal amounts in RNC-III and RNC-IV. E75 is 1% of p105HER-2. The amount of p138HER-2 was twice higher in RNC-III than in RNC-IV, E75 is 0.73% of p138. The amount of p155HER-2 was five times higher in RNC-III than in RNC-IV. E75 is 0.64% of p155. It is unlikely that any of them constituted pCE for E75 because RNC-IV induced more E75-TCR+ cells than RNC-III did. Furthermore, the overall amount

Figure 5. RNC-IV is a more efficient activator of the proliferation and differentiation of CD8+ E75-TCR+ IFN-\(\gamma\)-cells than RNC-III is. The responders shown are intermediate (A) and large lymphocytes (B). Cell size is indicated by the forward-scatter values. The number of E75-TCR+IFN-\(\gamma\)-cells was determined after 7 days' stimulation with Donor-3 DC pulsed with the indicated amounts of RNA and the corresponding amounts of RNA-bound proteins. No IL-2, indicates that IL-2 was not added to unstimulated PBMC-3. B, indicates that PBMC-3 were stimulated with IL-2 but not with RNAcs. C, E75-TCR+immunized cells activated with RNC-IV; C, E75-TCR+immunized cells activated with RNC-III. *Significantly higher numbers of E75-TCR+immunized were found in RNC-IV-stimulated cells (stimulation index >2.0, P <0.05) than in RNC-III-stimulated cells. C, RNC-IV contains higher amounts of p91 and p53HER-2, whereas RNC-III contains higher amounts of ubiquitin\(n\)\()\)-linked HER-2 fragments of 103, 138, and 155 kDa. RNC-III and RNC-IV were digested with Iso-T in the same experiment. HER-2 polypeptides in the Iso-T lysates were detected with mAb-19 and quantified by scanning densitometry. Iso-T digestion compared with no digestion shows (a) increase in p91HER-2 in RNC-IV after iso-T digestion and (b) decrease in p138 and p155 in RNC-IV compared with RNC-III. The total relative amount of E75, calculated as percentage of absorbance, was 74.7 (13.18 + 16.45 + 17.02 + 24.1 + 3.98) in RNC-III, whereas it was 79.1 (3.05 + 9.99 + 17.29 + 38.92 + 9.85) in RNC-IV.
of E75 in precursor peptides together (RNC-III, 74.8 relative absorbance; RNC-IV, 79.1 relative absorbance) was similar.

The 2.7 times stronger immunogenicity of RNC-IV paralleled the increase in the ratio of the amount of p53 in RNC-IV compared with that in RNC-III (2.48). The parallel suggests that p53HER-2 was the major pCE of E75. This finding is supported by the doubling of E75-TCR+ cells on stimulation with RIBO-17-Tx and the 2-fold to 3-fold increase of p53HER-2 in RIBO-17-Tx compared with RIBO-17 (Supplementary Fig. S1B and C).

RIBO-17-Tx, but not RIBO-17, is protected from terminal proteolytic degradation in vacuoles. To identify the pathway of ISMMC uptake and HLA class I-associated processing of ISMMC to E75, APC were treated with cytochalasin-D (a phagocytosis inhibitor), with CD91 and Lox-1 (blocking antibodies to the scavenger receptors), and with dansyl-cadaverine (Dan-C, an inhibitor of clathrin-mediated endocytosis; ref. 11). Cytochalasin-D did not inhibit but rather increased the uptake of immunogenic precursor of E75 by APC because more E75-TCR+ cells expanded in its presence than in its absence (Fig. 6A). Blocking CD91 inhibited proliferation of indicator E75-TCRmed+lo cells more than blocking of Lox-1 did (Fig. 6B).

To determine whether E75 was generated by proteasomal or nonproteasomal proteases, an E75 peptide–activated T-cell line was reactivated with APC pulsed with RNC-III in the presence and absence of the proteasome inhibitor MG132 and the cysteine-protease inhibitor E64. E64 is also a generic inhibitor of calpains and cathepsins G, D, L, and H. Treatment of APC with 10 μg of E64 increased by seven times the number of E75-TCRmed+lo cells activated by pCE from RNC-III compared with the unstimulated E75-TCR+ cells. MG132 inhibited proliferation in response to RNC-III (Supplementary Table S5). This finding confirmed that a substantial part of ISMMC is terminally degraded by cysteine proteases and is unavailable for processing to peptide by proteasome.

In the absence of chloroquine, RIBO-17-Tx was a 50% more efficient activator of TCRmed and TCRlo cells than RIBO-17. TCRmed and TCRlo cells need more TA for activation because they express less TCR than TCRhi cells. Chloroquine inhibited by 40% to 50% the proliferation of all indicator E75-TCR+ populations in response RIBO-17-Tx but enhanced by 30% the proliferation of E75-TCRmed+lo cells activated by RIBO-17 (Fig. 6C). Therefore, the pCE of E75 in RIBO-17-Tx, but not that in RIBO-17, was protected from terminal degradation in vacuoles.

Protection of RIBO-17-Tx from terminal proteolysis may be due to exhaustion of proteases during degradation of adducts by Taxol to ISMMC. Additional studies are needed to address whether the adducts brought by Taxol to ISMMC enhance the efficacy of TA processing and presentation.

Discussion

In ovarian tumor cells, we identified ISMMC that activated proliferation and partial differentiation of HER-2–specific CD8+ cells. Activation of proliferation by ISMMC did not require exogenous inflammatory cytokines, such as IL-12. ISMMC contained nascent TA/HER-2 polypeptide chains bound to synthesizing mRNA on ribosomes and linked to ubiquitin. The shortest and apparently most efficient pCE–HER-2 consisted of ubiquitin–45-kDa HER-2 (1–413) polypeptide. ISMMC were chaperoned by Gp96. Taxol-induced ISMMC contained the cochaperone Mpp-11J, which attracts Hsp70 to the ribosome.

Taxol-induced ISMMC were more efficient activators of E75-TCR+ cells than ISMMC in donors tested. Because Taxol quadrupled the amount of ISMMC relative to that in untreated SK-OV-3, we concluded that overall, Taxol-modified ISMMC can activate more T cells of various specificities than native ISMMC can.

ISMMC endowed with CD8+ cell activation and differentiation capacity have not been described in cancer or healthy cells. Activation of cellular immunity after phagocytosis of a molecular complex containing foreign antigen has been postulated to explain the signal that activated APC and the source of endogenous cytokines (21, 22). Our findings are supported by activation of...
B-cell autoimmunity by self–small mRNAs–nuclear proteins complexes (23, 24).

The self-mRNA sequence is considered an inefficient activator of IL-12 (25). Our RNC, tumor cells, and media were free of endotoxins, Mycoplasma, and bacteria. Therefore, it is unlikely that bacterial or viral RNA was introduced into the ISMMC. The IL-12–activating self-RNA originated from two possible sources: (a) the shortened poly(A)-chains of mRNA and (b) the longer HER-2 transcripts that contain the 3′-untranslated region (UTR) of HER-2 mRNA. Termination of translation is followed by activation of poly(A)–specific 3′-exocRNAse, which mediates deadenylation of mRNA to 15 to 30 residue-long poly(A) tails (26). The short poly(A)–chains activate TLRs (26). Transcription of 3′-UTR–HER-2 and enrichment of an 8-kb HER-2 transcript in SK-OV-3 (27–29) result in nucleotide chains of unique sequence. Such chains can activate TLRs or cytosolic RNA receptors in APC because they are seen as foreign RNA (30). However, heat shock proteins bound to RNAs can act as coactivators of RNA receptors by presenting some and masking other RNA sequences similar with tumor and viral peptides bound on peptidoglycans, which increase IL-6 production from iDC.10

Our findings suggest that TA from ISMMC is delivered to endosomes and reach the cytoplasm for proteasomal processing. This retrotranslocation pathway consists of transport of pCE through the route lysosomes → endosomes → ER → cytoplasm. The traffic of RNA-bound pCE from endosomes to the ER is facilitated by coat proteins, similar to the retrotranslocation of ricin A and Pseudomonas toxins (31, 32). The exit from the ER to the cytoplasm is facilitated by the one or two ubiquitin molecules linked to pCE–HER-2. Our hypothesis is supported by the immunogenicity of a fusion protein, influenza-neuraminidase-E75. The fusion protein was longer than expected, suggesting that it was posttranslationally glycosylated in the Golgi apparatus.11

Our results suggest a novel mechanism for the formation of immunogenic ISMMC. The “heavy” polyomies (RIBO-17/RIBO-17-Tx) are formed when translation stops in one ribosome. Other ribosomes attach to it, forming a large complex called “stalled ribosomes/polyomies” (17, 33). Because Iso-T treatment showed more p91HER-2 on RNC-IV than on RNC-III, the p91HER-2–synthesizing ribosome may have been stalled first. The small amounts of p155HER-2, p138HER-2, and p103HER-2 in RNC-IV relative to those in RNC-III indicate that more ribosomes synthesized p91HER-2 than p138HER-2 and p155HER-2 at the time of “stalling.”

RNC are transiently ubiquitin conjugated when protein synthesis is on “stand-by” and are deubiquitinated as synthesis restarts (34). A part of both p53HER-2 and p91HER-2 was polyubiquitinated. The ubiquitin-linked RNCs in an energetically transient state will be directed for cytoplasmic degradation when the energy of the system decreases or will be deubiquitinated when the ribosome is rescued (34). Such transient states of RNCs are easier to detect in stable proteins with long half-lives, such as HER-2 (t1/2 = 18 h; ref. 27).

ISMMC form much larger particles than individual ribosomes but require dissociation by RNaseA to activate T cells. The accumulated large ISMMC may undergo exocytosis even before tumor cell apoptosis. Such ISMMC are degraded to ribosomes by RNase in interstitial fluids or from APC (35, 36). ISMMC induce IL-12 in iDC and increase the number of CD40+ iDC.12 CD40 expression is associated with activation of CD8+ cells (30, 37). The pCE in ISMMC differs from the classic “defective-ribosomal product.” The proteasomal conjugation factor RAD23 is associated with RNC-I, which contains more cHER-2, and is absent from RNC-III and RNC-IV.13 The presence of RAD23 suggested that in normal conditions, RNC-I can be degraded with priority by the direct proteasomal pathway, whereas RNC-III and RNC-IV do not. However, because ISMMC are taken up by APC and directed to vacuoles, the direct proteasomal degradation pathway is either not accessed or, if accessed, the pCE of E75 is not in the optimal form for E75 peptide processing and presentation.

Taxol induces cellular autoimmunity in patients, such as scleroderma-like illness without antinuclear antibodies (38–40). Our findings indicate that quantitatively, Taxol-induced ISMMC expanded more CD8+ cells than natural ISMMC did. Qualitatively, stronger expansion by ISMMC was directed to cells expressing less TCR than cells that highly express TCR. The former need more peptide–MHC-I complexes than the latter. We do not know yet whether the amounts of ISMMC used induced TCR-activated death of TCRhi cells. ISMMC were considerably more efficient activators than peptides and mRNA. Identification of natural and Taxol-induced ISMMC protected from degradation indicated novel strategies for generating cancer vaccines with synthetic multimolecular complexes containing optimized components. Such vaccines should be useful after chemotherapy with Taxol in prevention of metastases.

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