Blocking the CD40–CD154 interaction is reported to be effective for transplantation management and autoimmune disease models in rodents and nonhuman primates. However, clinical trials with anti-CD154 mAbs were halted because of high incidence of thromboembolic complications. Thus, we generated and characterized a fully human anti-CD40 mAb ASKP1240, as an alternative to anti-CD154 mAb. In vitro ASKP1240 concentration-dependently inhibited human peripheral blood mononuclear cell proliferation induced by soluble CD154. In addition, ASKP1240 did not destabilize platelet thrombi under physiological high shear conditions while mouse anti-human CD154 mAb (mu5C8) did. And ASKP1240 itself did not activate platelet and endothelial cells. In vivo administration of ASKP1240 (1 or 10 mg/kg, intravenously) to cynomolgus monkeys, weekly for 3 weeks, significantly attenuated both delayed-type hypersensitivity and specific antibody formation evoked by tetanus toxoid. The immunosuppressive effect was well correlated with the CD40 receptor saturation. Thus, these results suggest that ASKP1240 is immunosuppressive but not prothromboembolic, and as such appears to be a promising therapeutic candidate for the management of solid organ transplant rejection and autoimmune diseases therapy.

Keywords: CD40, costimulation, immunosuppressive therapy, mAbs

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; DCs, dendritic cells; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FcγR, Fc gamma receptor; FITC, fluorescein isothiocyanate; geoMFI, geometric mean fluorescence intensity; GST, glutathione-S-transferase; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PD, pharmacodynamic; PE, phycoerythrin; PRP, platelet-rich plasma; shCD 154, soluble human CD154; TNF, tumor necrosis factor; TTx, tetanus toxoid

Received 26 November 2013, revised 14 January 2014 and accepted for publication 29 January 2014

Introduction

The CD40 molecule is mainly expressed on antigen-presenting cells such as macrophages, and dendritic cells (DCs) as well as on B lymphocytes and appears to play an important role in immunological responses (1). Blocking the CD40–CD154 interaction has shown therapeutic effects in several experimental disease models, including organ rejection after transplantation (2), atherosclerosis (3) and autoimmune diseases (4–7). Although several humanized anti-CD154 mAbs (hu5C8, IDEC-131 and ABI793) have been developed and shown to be markedly efficacious in nonhuman primate renal allograft models (8–12), there are significant obstacles to further clinical development. In particular, in early clinical trials with hu5C8 or IDEC-131 there were thromboembolic events (13–15). The mechanism is not fully understood (16–19), but recent studies have suggested that CD154 functions to stabilize arterial thrombi in a CD40-independent manner through its integrin binding KGD (Lys-Gly-Asp) sequence (20,21). It is assumed that targeting the CD40–CD154 pathway via CD40 rather than CD154 might allow an immunosuppressive effect,
while leaving the CD154–integrin interactions necessary to regulate thrombus stability unaltered. Several chimeric mAbs against CD40 (chi220 and chSD12) have been developed as alternatives to anti-CD154 mAbs and were also found to be effective in renal allograft models (22–24) as well as autoimmune disease models in nonhuman primates (25). However, these mAbs were immunogenic reducing their suitability for drug development.

Therefore, we generated a fully human anti-CD40 antagonistic mAb (ASKP1240) from trans-chromosome mice (26). This is an IgG4 masking antibody that shows neither antibody-dependent cell-mediated cytotoxicity (ADCC) nor complement-dependent cytotoxicity (CDC) (27). This ASKP1240 antibody was recently reported to significantly prolong kidney, liver and islet graft survival in nonhuman primates (28–31). The current study characterized this antibody with respect to its effects on soluble human CD154 (shCD154) induced cellular proliferation. In addition, the potential for prothromboembolic effects was assessed in vitro using human platelets and endothelial cells. Finally, the immunosuppressive activity and safety of ASKP1240 were examined in cynomolgus monkeys.

Materials and Methods

ASKP1240 antibody generation

Fully human anti-CD40 antibodies were generated using the KM mouse™ technology (26). These mice were immunized with soluble human extracellular domain CD40 protein and the splenocytes were fused with SP20 cells (ATCC, Rockville, MD). A SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA) was used for the cloning of the human antibody variable region. Human heavy and light chain variable sequences were subsequently cloned into IgG4 antibody expression vector. The expression vector was transfected into Chinese hamster ovary cells and the antibody ASKP1240 was expressed and purified.

ADCC assay

Blood samples were collected from human healthy volunteers and peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation. 51Cr-labeled Raji cells (ATCC) were incubated in triplicate with 100 μg/mL indicated antibodies and PBMCs at effector-target ratio of 100:1 at 37°C. After 4-h incubation, the radioactivity in the supernatants was counted. The percentage of specific lysis was calculated according to the following formula: % lysis = 100 × (ER – SR)/(MR – SR), where ER, SR and MR represent experimental, spontaneous and maximum 51Cr-release, respectively.

CDC assay

51Cr-labeled Raji cells were incubated with 100 μg/mL indicated antibodies and 10% normal human serum at 37°C. After 2-h incubation, the radioactivity in the supernatants was counted. The percentage of specific lysis was calculated as described above.

Internalization assay by flow cytometry

To measure the clearance of immunocomplexes from the cell surface, Ramos cells (ATCC) were incubated with fluorescein isothiocyanate (FITC)-labeled ASKP1240 or FITC-labeled anti-CD40 agonistic mAb (clone

G28.5; ATCC) in RPMI1640 supplemented with 10% fetal bovine serum (FBS) for 15 min at 4°C. The stained cells were washed, and then incubated for varying durations in culture medium at 37°C. At the given time points, cells were removed from the culture and washed with phosphate-buffered saline (PBS) containing 0.1% NaN3 to block internalization. Next, the cells were incubated with phycocerythrin (PE)-labeled anti-human IgG (BioLegend, San Diego, CA) or PE-labeled anti-mouse IgG (BioLegend) for 30 min at 4°C. The geometric mean fluorescence intensity (gMFI) was measured by flow cytometry and analyzed using CellQuest software (ver. 5.1.1; BD Biosciences, Franklin Lakes, NJ).

Cross-reactivity in the peripheral blood

Peripheral blood samples from mice (B6C3F1), rats (Sprague-Dawley), rabbits (Japanese White), cynomolgus monkeys and humans were incubated with FITC-labeled ASKP1240. The erythrocytes/plaquettes, lymphocyte, monocytes and granulocytes were identified by their light scatter characteristics and lineage-specific mAbs (anti-mouse CD3e, 145-2C11, BD; anti-CD45R/B220, RA3-6B2, BD; anti-mouse CD41, MVR350, BD; anti-rat CD3, G4.18, BD; anti-rat CD45RA, OK-33, BD; anti-rat CD42d, RPM-4, BD; anti-rabbit T lymphocyte, KEN-5, AbD Serotec, Kidlington, UK; anti-rabbit CD41, P2, Beckman Coulter, Brea, CA; anti-monkey CD3, SP34 BD; anti-monkey CD20, H29, Beckman Coulter; anti-mouse CD41a, H1P8, BD, anti-human CD3, UCHT1, Beckman Coulter; anti-human CD19, J4.119, Beckman Coulter; anti-human CD41, P2, BD). The fluorescent activity of each cell population was determined by flow cytometry.

Affinity analysis

CD40 complementary DNA clones were isolated from human, cynomolgus monkey and rabbit tissues. Glutathione-S-transferase (GST)-fused CD40 recombinant proteins were prepared using HEK293F cells. Affinity evaluation was carried out using a BiAcro 3000 (GE Healthcare, Piscataway, NJ). The flow cell of Sensor Chip CMS (GE Healthcare) was coupled with anti-GST antibody by amine coupling. The recombinant CD40 GST fusion proteins in HBS-EP buffer were injected at a flow rate of 10 μL/min, followed by ASKP1240 at concentrations of 0.16–10 μg/mL, flow rate of 10 μL/min. The association and dissociation reactions were recorded for 4 and 15 min, respectively. For regeneration, 10 mM glycine–HCl (pH 2.2) was injected at a 20 μL/min flow rate for 2 min. The kinetic parameters were calculated using BIAEVALUATION software version 4.0 (GE Healthcare).

PBMC proliferation

Three individual PBMC samples from each species (human, monkey and rabbit) were collected as described above and the cells were re-suspended in RPMI1640 supplemented with 10% FBS, and were cultured with 1 μg/mL Flag-tagged shCD154 (Alexis Biochemicals, Lausanne, Switzerland) and 10 μg/mL anti-Flag M2 mAb (Sigma-Aldrich, St. Louis, MO) to cross-link shCD154 in the presence or absence of various concentrations of ASKP1240 for 48 h at 37°C. After incubation, the cells were pulse labeled with [methyl-3H]-thymidine (1 μCi/well; GE Healthcare). Sixteen hours later the cells were harvested on microglass filter strips and the incorporated radioactivity was determined by liquid scintillation. Their ID50 was estimated using GraphPad Prism version 4.0 (GraphPad Software, La Jolla, CA).

Delayed-type hypersensitivity (DTH) reaction and antibody production in vivo

ASKP1240, at doses of 0 (placebo), 0.1, 1 and 10 mg/kg, was administered intravenously to 5 males/group on days 0, 7 and 14. For sensitization, the animals were anesthetized with ketamine then injected with 10 mL/tt of tetanus toxoid (TTx; Denka Seiken, Tokyo, Japan) vaccine into the skin on the back intradermally (50 μL/site x 12 sites) and thigh intramuscularly (0.6 mL/body) once on day 0. Three weeks later, 10 μL TTx solution, at a
concentration of 0, 1, 3 or 10 Lf/mL, was injected into the thoracic skin. Skin reactions at the injection sites were photographed and evaluated for erythematous and edematous reactions 48h after challenge. Reaction scores based on erythema and edema were determined according to the Draize skin test. The total score was the sum of the erythema and edema scores at an individual injection site. Anti-TTX IgG and IgM levels were determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated with TTx; then serially diluted monkey serum from the test animals was added. The plates were incubated at room temperature, and peroxidase-conjugated goat anti-monkey IgG or IgM antibodies (Abcam, Cambridge, MA) were added for detection. The titer was defined as the dilution magnitude of sera at which the ELISA optical density dropped to the control level.

**Receptor competition assay**

ASKP1240 was administered intravenously once at dose levels of 0 (placebo), 0.1, 1, 3 and 10 mg/kg to four male cynomolgus monkeys per group. Sampling points were set between days –11 and 49 according to dose level. The collected blood samples were lysed and stained with biotinylated ASKP1240 plus PE-labeled streptavidin (BD) and allophycocyanin-labeled anti-CD20 mAb (clone 2H7; BD). The geoMFI of PE in CD20+ lymphocytes was monitored by flow cytometry. ASKP1240 was also determined by an ELISA, as described before (28).

**Platelet preparation and activation**

Platelet-rich plasma (PRP) was generated by centrifugation citrated human blood from healthy donors at 150g for 10 min. Platelets were stimulated with 0.2 U/mL human thrombin (Sigma–Aldrich) at 37°C without stirring. After activation, platelets were fixed with 1% paraformaldehyde, washed and reacted with a platelet-specific PE-labeled anti-CD41 mAb (clone 2H7; DAKO, Carpinteria, CA) and FITC-labeled ASKP1240 or FITC-labeled mu5C8. The fluorescence was measured by flow cytometry.

**In vitro experiment on platelet thrombus formation**

Blood samples were collected from three healthy human volunteers and used within 1 h of blood collection. A silicon gasket with a flowpath height of 127 μm was placed between a parallel plate perfusion chamber (GlycoTech, Rockville, MD) and a human type III fibrillar collagen coated Petri dish (Sigma–Aldrich). Blood sample was divided into four tubes and added with 100 μg/mL indicated antibodies: ASKP1240, human isotype antibody, mu5C8 (ATCC) or mouse isotype antibody. The antibody-treated blood samples were aspirated through the chamber by a syringe pump at a constant flow stress to produce wall shear stresses of 500/s and 1500/s. Blood samples were aspirated through the chamber by a syringe pump at a constant flow stress to produce wall shear stresses of 500/s and 1500/s. Complete gross necropsies were performed on three males and three females from each group on day 28 (end of the dosing period). The remaining three males and three females in each group were monitored for an additional 4 weeks (end of the recovery period). The number of circulating CD20+ B cells was determined flow cytometrically. The anti-ASKP1240 titer was also measured by BLAcore as described previously (28).

**Animal welfare**

The experiments using cynomolgus monkeys were performed in facilities at Shin Nippon Biomedical Laboratories (SNBL; Kagoshima, Japan). This study was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethical criteria contained in the bylaws of the committee.

**Results**

**Cytotoxicity of ASKP1240**

The constant region of ASKP1240 is converted to IgG4 to reduce ADCC and CDC activities that might injure CD40 positive cells (i.e. B cells, monocytes, platelet, endothelial cells, etc.). One hundred micrograms per milliliter of ASKP1240 IgG4 did not induce CD40 expressing cell lysis via ADCC and CDC activity in contrast with rituximab and IgG1 version of ASKP1240 (Figure 1).

![Figure 1: Cytotoxic assay of ASKP1240. Antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities of 100 μg/mL of antibodies were evaluated by chromium release assay. The percentage of specific lysis of B-lymphoma cells (Raji) is represented as the mean ± SD of three wells.](image-url)
**Internalization assay of ASKP1240**

Since CD40 ligation can lead to internalization of cell surface CD40 (32), a determination was made as to whether ASKP1240 can elicit internalization of CD40. In this experiment, internalized antigen showed FITC fluorescence only, but residual cell surface antigen showed both PE and FITC fluorescence. Although binding of the anti-CD40 agonistic mAb, G28.5, leads to an immediate decrease in PE fluorescence, ASKP1240 affected neither FITC nor PE fluorescence levels (Figure 2).

**Cross-reactivity and binding affinity of ASKP1240**

The cross-reactivity of ASKP1240 to mouse, rat, rabbit, cynomolgus monkey or human CD40 on blood cells was assessed flow cytometrically. Specific binding of FITC-labeled ASKP1240 was detected in human, cynomolgus monkey and rabbit CD40, indicating that the cynomolgus monkey and rabbit have the potential to be pharmacologically reactive to ASKP1240. The ASKP1240 reactive blood cell populations were also identified (i.e. B cells, monocytes and platelets) (Table 1). Equilibrium and kinetic-binding analyses were performed on ASKP1240 using surface plasmon resonance. The ASKP1240 molecule has approximately sixfold higher avidity (KD) for human and cynomolgus monkey CD40 than for rabbit CD40. Kinetic binding analysis revealed the binding rates (ka) of ASKP1240 for human, cynomolgus monkey and rabbit CD40 were similar, though dissociation rates (kd) were not. The dissociation rates of ASKP1240 for human and cynomolgus monkey CD40 were approximately sixfold slower than that for rabbit CD40 (Table 2).

**Effect of ASKP1240 on PBMC proliferation induced by shCD154**

The effect of ASKP1240 on PBMC proliferation induced by shCD154 was compared using human, cynomolgus monkey and rabbit blood samples. The ASKP1240 mAb inhibited PBMC proliferation induced by shCD154 in a concentration-dependent manner in all three species with IC50 estimated to be 5.8, 25.1 and 91.8 ng/mL, for human, monkey and rabbit samples, respectively (Figure 3).

**Effects of ASKP1240 on DTH reactions and anti-TTx antibody productions in cynomolgus monkeys in vivo**

The ASKP1240 inhibited DTH reaction in a dose-dependent manner (Figure 4A). Histopathologically, vasculitis and inflammatory cell infiltration including mononuclear cells and acidophils were observed in the dermis and subcutis at the challenge sites injected with TTx in the control group, but not at the sites injected with physiological saline. The incidences and degrees of histological changes decreased dose dependently (data not shown). Anti-TTx antibodies were also assessed over time, through day 23. ASKP1240

---

**Figure 2: Modulation of CD40 antigens on B cell lines analyzed by flow cytometry.** Ramos cells were incubated with FITC-labeled ASKP1240 (A) or FITC-labeled G28.5 (B) in culture medium at 37°C. At the given time points, cells were washed with PBS containing NaN3 and then labeled with PE-labeled secondary antibodies. The fluorescence of FITC represents simultaneous measurements of cell surface and internalized immunocomplexes; while that of PE represents only cell surface immunocomplexes. The percentage of geoMFI at indicated time point relative to prevalue is plotted over time. FITC, fluorescein isothiocyanate; geoMFI, geometric mean fluorescence intensity; PBS, phosphate-buffered saline; PE, phycoerythrin.
dose-dependently inhibited anti-TTx IgG and anti-TTx IgM productions (Figure 4B and C) with complete inhibition in antibody formation seen at doses of 10 mg/kg.

**Pharmacokinetics and receptor occupancy**

After single administration, the serum ASKP1240 concentrations increased dose dependently, and decreased rapidly in the 0.1 mg/kg group, and slowly in the 1, 3 and 10 mg/kg groups (Figure 5A). The receptor occupancy was monitored as a pharmacodynamic (PD) marker. The binding of biotinylated ASKP1240 completely diminished just after the administration of ASKP1240. Although the free CD40 receptor recovered immediately in 0.1 mg/kg group, 1, 3 and 10 mg/kg sustained full receptor occupancy for 5, 10 and 14 days, respectively. The recovery of free CD40 receptor also delayed in a dose-dependent manner (Figure 5B). The CD40 antigen was almost completely occupied when the serum ASKP1240 concentrations were \( \geq 100 \text{ng/mL} \) (Figure 5C).

**Influence of anti-CD40 and anti-CD154 antibodies on human platelet thrombus formation in vitro**

Flow cytometric analysis showed that ASKP1240 bound to both unstimulated and stimulated platelets. Conversely, the mouse anti-human CD154 mAb, mu5C8, bound only thrombin-stimulated platelets (Figure 6A). A recent study

---

**Table 2:** Summary of equilibrium and apparent kinetic constants

| Immobilized protein | Analyte       | \(ka (\times 10^5), \text{M}^{-1} \text{s}^{-1}\) | \(kd (\times 10^{-4}), \text{s}^{-1}\) | \(KD, \text{nM}\) |
|---------------------|---------------|-----------------------------------------------|-----------------------------------|-----------------|
| Human CD40          | ASKP1240      | \(1.22 \pm 0.12\)                             | \(0.29 \pm 0.01\)                 | \(0.24 \pm 0.02\) |
| Monkey CD40         | ASKP1240      | \(1.26 \pm 0.03\)                             | \(0.36 \pm 0.03\)                 | \(0.28 \pm 0.03\) |
| Rabbit CD40         | ASKP1240      | \(1.32 \pm 0.03\)                             | \(1.90 \pm 0.33\)                 | \(1.44 \pm 0.26\) |

\(ka\), association constant; \(kd\), dissociation constant; \(KD\), equilibrium dissociation constant.
The mean \( \pm \text{SD} \) of three independent experiments is shown.

---

Figure 3: Inhibition of immune cell activation induced by shCD154 in vitro. Inhibition of PBMC proliferation stimulated with shCD154 in human, cynomolgus monkey and rabbit samples. The effects of increasing the concentration of ASKP1240 were evaluated. Cell proliferation was measured by incorporation of \(^{3}H\)-thymidine. The percentage of proliferation relative to the ASKP1240 (-) control is plotted as the mean \( \pm \text{SE} \) of three individuals. PBMC, peripheral blood mononuclear cell; shCD154, soluble human CD154.

Figure 4: Inhibition of DTH reactions and antibody productions in vivo. (A) Animals were immunized once with TTx on day 0 and challenged with injections into the thoracic skin of serially diluted TTx on day 21. ASKP1240 or placebo was administered intravenously on days 0, 7 and 14. At 48h after challenge, skin reactions at the injection site were scored as described in the Materials and Methods section. The DTH scores are expressed as the mean \( \pm \text{SE} \) of five individuals. (B, C) Animals were immunized once with TTx on day 0 and blood samples were obtained twice per week for serum titration. Anti-TTx IgG (B) and anti-TTx IgM (C) titers were measured by ELISA. The IgG and IgM titers are expressed as the means \( \pm \text{SE} \) of five individuals. DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; TTx, tetanus toxoid.
indicated that CD154 contributes to the stabilization of the formed platelet thrombi via an αIIbβ3-dependent (CD40 independent) mechanism (20). Indeed, although blood from CD154-deficient mice shows normal hemostasis processes under low shear stress conditions, the formed thrombi frequently ruptured and embolized under high shear stress conditions. Therefore, the influence of anti-CD154 and ASKP1240 on the platelet thrombus stability was evaluated by a perfusion assay. Under low shear stress condition (500/s), ASKP1240 and mu5C8-treated blood samples formed normal platelet thrombi on the collagen-coated surface. However, under high shear stress condition (1500/s) only smaller thrombi remained in mu5C8-treated blood sample, though normal platelet thrombi were observed in ASKP1240-treated blood sample (Figure 6B).

**Influence of ASKP1240 on human platelet and endothelial cell activation markers**

Since some anti-CD40 mAbs and CD154 reportedly activate platelets and vascular endothelial cells (33–35), we evaluated the influence of ASKP1240 on these cells. Positive control reagent (SFLLRN or TNFα) and shCD154 up-regulated cell activation markers, such as CD62P (P-selectin), CD62E (E-selectin) and CD142 (tissue factor). On the other hand, ASKP1240 by itself did not up-regulate their activation markers, and rather inhibited cell activation by shCD154 (Figure 7).
Repeated dose safety assessment
No clinical signs or other abnormalities including thrombo-
sis-related events were observed during dosing, at 1 week following the end of the dosing, or at the end of
the 4-week recovery period. No ASKP1240-related changes
were noted in body weight, food consumption, urinalysis,
hematology or blood chemistry measurements in any group
(data not shown). There were no significant decreases in the
circulating B lymphocytes counts (Figure 8). During the
study period, no evidence suggesting the production of anti-
ASKP1240 antibodies was noted in any animal in any group
at any measurement point (data not shown).

Discussion

T cell activation is a multistep process involving T cell
receptor engagement by antigen/major histocompatibility
complex as well as involvement of costimulatory molecules.
Two of the best characterized costimulatory pathways are
the interactions between B7-CD28 and CD40–CD154.
Blockade of these pathways has exhibited extraordinary
efficacy in several transplantation and autoimmune disease
models (2–12). B7-CD28 blockers, such as abatacept and
belatacept, are in fact now on the market. On the other
hand, the development of CD40–CD154 blockers has not
made substantial progress. The main reason is that several
anti-CD154 mAbs (5C8, IDEC-131 and ABI793) showed
unexpected high incidence of agent-associated thrombo-
embolic complications (13–15). As alternatives, mAbs
against CD40 (chi220 and ch5D12) have been developed.
These mAbs were effective and no thromboembolic events
were reported in nonhuman primate models of either organ
transplantation or autoimmune disease. However, chi220,
IgG1 with high cytotoxicity, reduced the number of
circulating B cells and was associated with cytomegalovirus
infection. Furthermore, both mAbs are chimeric and have
proven to be highly immunogenic (22,23). Thus, in order to
circumvent this problem, we generated an antagonistic fully
human IgG4 anti-CD40 mAb, ASKP1240, in trans-chromo-
some mice (26). Our rationale was as follows: (1) a fully
human antibody is expected to have low immunogenicity,
(2) IgG4 has a masking property, without depleting CD40
positive cells, such as B cells, DCs, macrophages, platelets,
etc. and (3) IgG4 has low binding affinity for FC gamma
receptors (FcyRs), which can cross-link the antibodies and
induce agonistic activities (36). Therefore, ASKP1240 is
expected to exert its immunomodulatory action without the

Figure 7: Influence of ASKP1240 on human platelet and endothelial cell activation markers. (A) Effect of ASKP1240 on platelet
P-selectin (CD62P) expression. Diluted PRP was incubated with indicated reagents. Shown are dot plots obtained from flow cytometry
analyses demonstrating the cells incubated only media, 100 μM of thrombin receptor activating peptide (SFLLRN), 1 mg/mL of ASKP1240,
1 μg/mL of shCD154 or 1 mg/mL of ASKP1240 plus 1 μg/mL of shCD40. (B) Effect of ASKP1240 on endothelial cell E-selectin (CD62E) and
tissue factor (CD142) expression. Shown are dot plots obtained from flow cytometry analyses demonstrating the cells incubated with only
media, 100 U/mL of TNFα, 1 mg/mL of ASKP1240, 1 μg/mL of shCD154 or 1 mg/mL of ASKP1240 plus 1 μg/mL of shCD40. PRP, platelet-rich
plasma; shCD154, soluble human CD154; TNF, tumor necrosis factor.
unwanted effects of anti-drug antibody emergence or the depletion of CD40 positive cells.

It is reported that costimulatory blocker with higher avidity resulted in more potent efficacy (37). The ASKP1240 mAb was shown to bind to human CD40 with high affinity (KD = 0.24 nM) and to effectively block the interaction of CD40 with CD154. Its binding activity and blocking potency for human and cynomolgus monkey CD40 were similar.

In the in vivo DTH and antibody production models, weekly administration of ASKP1240 at dose of 10 mg/kg, completely suppressed both the cell-mediated and antibody immune responses, while weekly administration of 1 mg/kg exerted a suboptimal effect. This is reasonable considering the result of receptor competition assay and indicates that receptor occupancy can be used as PD marker of ASKP1240. Although free CD40 antigen started to increase from around 5 days after the administration in 1 mg/kg group, it is expected that weekly administration of 10 mg/kg sustained complete receptor occupancy during this study. Certainly the amount of free CD40 antigen in 1 mg/kg would be very few, but we might have to aware of the accessibility of ASKP1240 for precise pharmacological prediction. Peripheral blood cell would be the easier target for antibody drug to access compared to lymph nodes and tissues. From the pharmacokinetic/PD study, the peripheral CD40 antigens were found to be almost completely occupied when the serum ASKP1240 concentrations were ≥100 ng/mL, and were maintained at a nadir when the serum ASKP1240 concentrations were ≥1000 ng/mL.

In the safety study, the weekly administration of ASKP1240 at doses up to 100 mg/kg did not lead to significant reduction in the number of circulating B cells or to any adverse events, including thrombosis. Anti-ASKP1240 antibodies were not detected in any animal in any group at any measurement point. Previous studies using mAbs directed against CD154 were associated with an increased risk of thromboembolic complications. Although the mechanism remains controversial, it was reported that shCD154 may stabilize arterial thrombi in vivo by an \( \alpha_{II} \beta_{3} \)-dependent (CD40-independent) mechanism via outside-in signaling through its integrin binding KGD sequence (20). Furthermore, CD154-deficient mice have been shown to form unstable thrombi, whereas this defect is not observed in CD40-deficient mice (20,37). Instability of thrombi can be a risk factor for thromboembolic complications because it occurs when formed thrombi detach from blood vessels and circulate as emboli. The present study showed that ASKP1240 did not affect the platelet thrombus formation nor did it destabilize platelet thrombi under high-flow condition. Conversely, while mu5C8 did not inhibit platelet thrombus formation on collagen under low-flow condition, it did destabilize it under high-flow condition. Some investigators have speculated that thromboembolic...
complications by anti-CD154 mAbs are attributable to platelet activation via platelet FcγR or complement deposition, rather than the αIIbβ3-dependent mechanism (16). Even if this is the case, ASKP1240 is expected to be safe because prethrombotic anti-CD154 mAbs, such as hu5C8, IDEC-131 and AB1793, are comprised of recombinant IgG1, while ASKP1240 is IgG4, which has an extremely lower affinity for platelet FcγR and extremely lower complement activation capacity than IgG1 (27). In addition, although some anti-CD40 agonistic mAbs and CD154 reportedly act on platelets and vascular endothelial cells to up-regulate their activation markers, such as P-selectin, E-selectin and tissue factors (33–35), ASKP1240 by itself did not up-regulate these markers. In addition to these in vitro and in vivo studies presented here, ASKP1240-related thrombosis was not observed among 72 renal transplantation recipient monkeys treated with ASKP1240. These experimental data, taken together, suggest that ASKP1240 itself is not inherently prothromboembolic.

In conclusion, the novel fully human antagonistic anti-CD40 mAb, ASKP1240, inhibited shCD154-stimulated cellular proliferation in vitro demonstrating its ability to block CD40–CD154 interactions on a cellular level. In vivo, ASKP1240 administration suppressed indicators of both cell-mediated and antibody immune responses (DTH and anti-TTx antibody responses). In vitro platelet studies suggest that ASKP1240 is not prothromboembolic in that it did not inhibit collagen-induced stable thrombus formation nor did it destabilize the thrombus once formed under flow conditions. Finally, the safety studies showed that the weekly administration of ASKP1240 at doses up to 100 mg/kg was not immunogenic, and resulted in no significant toxicity. Based on these findings, it is concluded that the fully human antagonistic CD40 mAb, ASKP1240, is a good candidate for further clinical development in transplant and autoimmune diseases.

Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. Y. Miyao, S. Sakuma and F. Kinugasa are employees of Astellas Pharma, Inc.

References

1. van Kooten C, Banchereau J. CD40–CD154 ligand. J Leukoc Biol 2000; 67: 2–17.
2. Nathan MJ, Yin D, Eichwald EJ, Bishop DK. The immunobiology of inductive anti-CD40L therapy in transplantation: Allograft acceptance is not dependent upon the depletion of graft-reactive T cells. Am J Transplant 2002; 2: 323–332.
3. Remskar M, Li H, Chyu KY, Shah PK, Cercek B. Absence of CD40 signaling is associated with an increase in intimal thickening after arterial injury. Circ Res 2001; 88: 390–394.
4. Daiikh DI, Finck BK, Linsley PS, Hollenbaugh D, Wofsy D. Long-term inhibition of murine lupus by brief simultaneous blockade of the B7/CD28 and CD40/gp39 costimulation pathways. J Immunol 1997; 159: 3104–3108.
5. Gerrits K, Laman JD, Noelle RJ. et al. CD40–CD154 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. Proc Natl Acad Sci USA 1996; 93: 2499–2504.
6. Durie FH, Fava RA, Foy TM, Aruffo A, Ledbetter JA, Noelle RJ. Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. Science 1993; 261: 1328–1330.
7. Liu Z, Geboes K, Colpaert S, et al. Prevention of experimental colitis in SCID mice reconstituted with CD48R8hig CD4+ T cells by blocking the CD40–CD154 interactions. J Immunol 2000; 164: 6005–6014.
8. Kirk AD, Burkly LC, Batty DS, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. Nat Med 1999; 5: 686–693.
9. Kirk AD, Harlan DM, Armstrong NN, et al. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. Proc Natl Acad Sci USA 1997; 94: 8789–8794.
10. Preston EH, Xu H, Dhanireddy KK, et al. IDEC-131 (anti-CD154), sirolimus and donor-specific transfusion facilitate operational tolerance in non-human primates. Am J Transplant 2005; 5: 1032–1041.
11. Schuler W, Bigaud M, Brinkmann V, et al. Efficacy and safety of AB1793, a novel human anti-human CD154 monoclonal antibody, in cynomolgus monkey renal allotransplantation. Transplantation 2004; 77: 717–726.
12. Kanmaz T, Fechner JJ Jr, Torrealba J, et al. Monotherapy with the novel human anti-CD154 monoclonal antibody AB1793 in rhesus monkey renal transplantation model. Transplantation 2004; 77: 914–920.
13. Knechtle SJ, Hamawy MM, Hu H, Fechner JH Jr, Cho CS. Tolerance and near-tolerance strategies in monkeys and their application to human renal transplantation. Immunol Rev 2001; 183: 205–213.
14. Vincenti F. What’s in the pipeline? New immunosuppressive drugs in transplantation. Ann Transplant 2002; 2: 898–903.
15. Larsen CP, Knechtle SJ, Adams A, Pearson T, Kirk AD. A new look at blockade of T-cell costimulation: A therapeutic strategy for long-term maintenance immunosuppression. Am J Transplant 2006; 6: 876–883.
16. Prasad KS, Andre P, Yan Y, Phillips DR. The platelet CD40L/GP IIb–IIIa axis in atherothrombotic disease. Curr Opin Hematol 2003; 10: 356–361.
17. Langer F, Ingersoll SB, Amirkhosravi A, et al. The role of CD40 in CD40L- and antibody-mediated platelet activation. Thromb Haemost 2005; 93: 1137–1146.
18. De Reys S, Blom C, Lepoudre B, et al. Human platelet aggregation by murine monoclonal antplatelet antibodies is subtype-dependent. Blood 1993; 81: 1792–1800.
19. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. Nat Med 2000; 6: 114.
20. Andre P, Prasad KS, Denis CV, et al. CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. Nat Med 2002; 8: 247–252.
21. Prasad KS, Andre P, He M, Bao M, Manganello J, Phillips DR. Soluble CD40 ligand induces beta3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. Proc Natl Acad Sci USA 2003; 100: 12367–12371.
22. Haanstra KG, Ringers J, Sick EA, et al. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. Transplantation 2003; 75: 637–643.
23. Pearson TC, Trambley J, Odom K, et al. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. Transplantation 2002; 74: 933–940.

24. Adams AB, Shirasugi N, Jones TR, et al. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. J Immunol 2005; 174: 542–550.

25. Laman JD, ’t Hart BA, Brok H, et al. Protection of marmoset monkeys against EAE by treatment with a murine antibody blocking CD40 (mu5D12). Eur J Immunol 2002; 32: 2218–2228.

26. Ishida I, Tomizuka K, Yoshida H, et al. Production of human monoclonal and polyclonal antibodies in TransChromo animals. Cloning Stem Cells 2002; 4: 91–102.

27. van der Pol W, van de Winkel JG. IgG receptor polymorphisms: Risk factors for disease. Immunogenetics 1998; 48: 222–232.

28. Imai A, Suzuki T, Sugitani A, et al. A novel fully human anti-CD40 monoclonal antibody, ASKP1240, for kidney transplantation in cynomolgus monkeys. Transplantation 2007; 84: 1020–1028.

29. Aoyagi T, Yamashita K, Suzuki T, et al. A human anti-CD40 monoclonal antibody, ASKP1240, for kidney transplantation in cynomolgus monkeys: Induction and maintenance therapy. Am J Transplant 2009; 9: 1732–1741.

30. Oura T, Yamashita K, Suzuki T, et al. Long-term hepatic allograft acceptance based on CD40 blockade by ASKP1240 in nonhuman primates. Am J Transplant 2012; 12: 1740–1754.

31. Watanabe M, Yamashita K, Suzuki T, et al. ASKP1240, a fully human anti-CD40 monoclonal antibody, prolongs pancreatic islet allograft survival in nonhuman primates. Am J Transplant 2013; 13: 1976–1988.

32. Press OW, Farr AG, Borroz Ki, Anderson SK, Martin PJ. Endocytosis and degradation of monoclonal antibodies targeting human B-cell malignancies. Cancer Res 1989; 49: 4910–4912.

33. Inwald DP, McDowall A, Peters MJ, Callard RE, Klein NJ. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. Circ Res 2003; 92: 1041–1048.

34. Slupsky JR, Kalbas M, Willuweit A, Henn V, Kroczen RA, Muller-Berghaus G. Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. Thromb Haemost 1998; 80: 1008–1014.

35. Miller DL, Yaron R, Yellin MJ. CD40L–CD40 interactions regulate endothelial cell surface tissue factor and thrombomodulin expression. J Leukoc Biol 1998; 63: 373–379.

36. Xu Y, Szalai AJ, Zhou T, et al. Fc gamma Rs modulate cytotoxicity of anti-Fas antibodies: Implications for agonistic antibody-based therapeutics. J Immunol 2003; 171: 562–568.

37. Crow AR, Leytin V, Starkey AF, Rand ML, Lazarus AH. CD154 (CD40 ligand)-deficient mice exhibit prolonged bleeding time and decreased shear-induced platelet aggregates. J Thromb Haemost 2003; 1: 850–852.