lymphopoietin expression in parallel with a reduction in serine protease activity and elevation in β-glucocerebrosidase activity (Figures 1 and 2, and Supplementary Figures S1 and S2), suggesting not only that coaplication of PAR2 antagonist and LBA could be involved in both PAR2-independent and PAR2-dependent mechanisms, but also that it might be essential to account for both mechanisms to confer significant therapeutic benefits in AD. Meanwhile, the antipruritic effect from inhibiting PAR2 signaling also could be involved in the therapeutic effects, although we could not evaluate quantitatively the degree of itch in this study.

This study demonstrates that coaplications of a PAR2 antagonist and the polyhydroxy acid LBA could represent a novel therapeutic strategy that simultaneously addresses the two mechanisms of AD pathogenesis, namely, skin barrier abnormality and allergic inflammation. The study results form the basis for further evaluation of this strategy in other AD animal models and in human AD.

All experiments with mice were approved by the Ethics of Animal Experimentation Committee of Oita University.

CONFLICT OF INTEREST
SK Jeong is an employee of NeoPharm Co., Ltd., South Korea.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.11.011.

**REFERENCES**


See related commentary on pg 362

**Melanoma-Directed Activation of Apoptosis Using a Bispecific Antibody Directed at MCSP and TRAIL Receptor-2/Death Receptor-5**

TO THE EDITOR

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an immune effector protein that induces apoptosis in virus-infected and cancer cells by activating death receptor-4 (DR4) and/or death receptor-5 (DR5) without deleterious activity toward DR4/DR5-expressing normal cells (Ashkenazi et al., 2008). Consequently, DR4/DR5 agonists are promising anticancer agents. Treatment with “first-generation” DR4/DR5-targeted therapeutics, such as recombinant
Y He et al.
Melanoma-Directed Activation of Death Receptor 5

Figure 1. Melanoma-associated chondroitin sulfate proteoglycan (MCSP)-directed apoptotic activity of MCSPxDR5 toward melanoma cells. (a) Schematic diagram of the tetravalent bispecific antibody MCSPxDR5. (b) SK-MEL-28 (MCSP+) and (c) DLD1 (MCSP-) cancer cells were incubated with MCSPxDR5 (250 ng/ml) in the presence or absence of anti-MCSP antibody (mAb 9.9.27; 10 μg/ml). Next, cells were stained using a polyclonal phycoerythrin (PE)-conjugated goat anti-mouse antibody and analyzed by flow cytometry. (d) MCSP+ melanoma cell lines and MCSP- colorectal cancer cell line DLD-1 were preincubated with MCSPxDR5 (2.5 μg/ml) or medium at 4 °C for 40 minutes, followed by 2 washes with excess cold phosphate buffered saline. Subsequently, cells were incubated for 18 hours at 37 °C/5% CO2, after which apoptosis was measured by flow cytometry using annexin V-FITC/propidium iodide (PI) staining. (e) MCSP+ and MCSP- cancer cell lines were treated with MCSPxDR5 (1.0 μg/ml) or left untreated, after which apoptosis was measured after 18 hours as in d. (f) MCSP+ and MCSP- cancer cell lines were treated with MCSPxDR5 (1.0 μg/ml) or left untreated, after which cell viability was determined by a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) viability assay after 72 hours. Viability after MCSPxDR5 treatment was calculated as percentage of medium control. (g) MCSP+/DR5+ cell lines MM-RU, SK-MEL-28, and A375M were treated with MCSPxDR5 (250 ng/ml) or left untreated in colony-forming agar assays for 72 hours, after which the number of colonies was determined by counting three fields of view in triplicate. Number of colonies was represented as percentage of colonies in medium control. (h) Representative light microscopic pictures of colony size of A375M cells in medium control versus MCSPxDR5-treated conditions in colony-forming assay and dose-response curve of colony size upon MCSPxDR5 treatment. Bar = 100 μm. (i) After approval of the University Medical Center Groningen ethics review board and written informed consent, primary patient-derived melanoma cells were obtained from surgical waste. Primary melanoma cells (n = 11, used before passage 4) were treated with MCSPxDR5 (1.0 μg/ml) or left untreated for 18 hours, after which apoptosis was analyzed by flow cytometry using annexin V-FITC staining. Statistical analysis was performed using two-sided unpaired Student t test. Statistical analysis of primary patient-derived cultures in i was performed using the Mann-Whitney U test. *P < 0.05; ***P < 0.001. n.s., not significant.

Figure 1. Melanoma-associated chondroitin sulfate proteoglycan (MCSP)-directed apoptotic activity of MCSPxDR5 toward melanoma cells. (a) Schematic diagram of the tetravalent bispecific antibody MCSPxDR5. (b) SK-MEL-28 (MCSP+), and (c) DLD1 (MCSP-) cancer cells were incubated with MCSPxDR5 (250 ng/ml) in the presence or absence of anti-MCSP antibody (mAb 9.9.27; 10 μg/ml). Next, cells were stained using a polyclonal phycoerythrin (PE)-conjugated goat anti-mouse antibody and analyzed by flow cytometry. (d) MCSP+ melanoma cell lines and MCSP- colorectal cancer cell line DLD-1 were preincubated with MCSPxDR5 (2.5 μg/ml) or medium at 4 °C for 40 minutes, followed by 2 washes with excess cold phosphate buffered saline. Subsequently, cells were incubated for 18 hours at 37 °C/5% CO2, after which apoptosis was measured by flow cytometry using annexin V-FITC/propidium iodide (PI) staining. (e) MCSP+ and MCSP- cancer cell lines were treated with MCSPxDR5 (1.0 μg/ml) or left untreated, after which apoptosis was measured after 18 hours as in d. (f) MCSP+ and MCSP- cancer cell lines were treated with MCSPxDR5 (1.0 μg/ml) or left untreated, after which cell viability was determined by a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) viability assay after 72 hours. Viability after MCSPxDR5 treatment was calculated as percentage of medium control. (g) MCSP+/DR5+ cell lines MM-RU, SK-MEL-28, and A375M were treated with MCSPxDR5 (250 ng/ml) or left untreated in colony-forming agar assays for 72 hours, after which the number of colonies was determined by counting three fields of view in triplicate. Number of colonies was represented as percentage of colonies in medium control. (h) Representative light microscopic pictures of colony size of A375M cells in medium control versus MCSPxDR5-treated conditions in colony-forming assay and dose-response curve of colony size upon MCSPxDR5 treatment. Bar = 100 μm. (i) After approval of the University Medical Center Groningen ethics review board and written informed consent, primary patient-derived melanoma cells were obtained from surgical waste. Primary melanoma cells (n = 11, used before passage 4) were treated with MCSPxDR5 (1.0 μg/ml) or left untreated for 18 hours, after which apoptosis was analyzed by flow cytometry using annexin V-FITC staining. Statistical analysis was performed using two-sided unpaired Student t test. Statistical analysis of primary patient-derived cultures in i was performed using the Mann-Whitney U test. *P < 0.05; ***P < 0.001. n.s., not significant.
cell surface of >90% of melanomas and is a well-established target for melanoma immunotherapy because its expression in normal tissues is mainly restricted to melanocytes (Campoli et al., 2004). Correspondingly, MCSPxDR5 strongly bound to MCSP\(^+\) SK-MEL-28 melanoma cells (Figure 1b, red line vs. black line) and minimally to MCSP\(^+\)/DR5\(^+\) DLD-1 carcinoma cells (Figure 1c, red line vs. black line). Binding of MCSPxDR5 to SK-MEL-28 was abrogated by pre-incubation with MAb 9.2.27 (Figure 1b, blue line). Thus, MCSPxDR5 binds to cell surface-expressed DR5, but binding to melanoma cells as such is dominated by its high-affinity for MCSP.

Treatment with MCSPxDR5 for 40 minutes at 4 °C followed by removal of unbound antibody selectively triggered apoptosis in MCSP\(^+\) and DR5\(^+\) melanoma cell lines MM-RU, M14, MCSP, and A375M but not in MCSP\(^+\) HCT116 carcinoma cells (Figure 1d). Of note, treatment in the continued presence of MCSPxDR5 also induced apoptosis in DLD-1 cells, although to a markedly lower extent (Figure 1e). Furthermore, MCSPxDR5 treatment strongly reduced tumor cell viability (Figure 1f) and anchorage-independent colony-forming capacity of melanoma cells (Figure 1g). Any residual colonies formed during treatment were reduced in size >90% (Figure 1h). Importantly, primary tumor cells isolated from melanoma patients proved sensitive to MCSPxDR5 treatment, with a >40% increase in apoptosis compared to medium control (Figure 1i).

Because therapeutic activity of agonistic DR5 antibodies such as tigatuzumab appears to require cross-linking by Fc receptors of myeloid effector cells, we evaluated whether MCSP-directed activity of MCSPxDR5 was also enhanced by cross-linking of its Fc domain. We exploited MCSP\(^+\)/DR5\(^+\) Jurkat cells as indicator cells that are sensitive only to secondary cross-linked MCSPxDR5. As expected, treatment of Jurkat cells with MCSPxDR5 alone did not induce apoptosis (Figure 2a). However, cross-linking of MCSPxDR5 using an anti-human Fc antibody triggered apoptosis >95% (Figure 2a). This enhancement was already apparent at low MCSPxDR5 doses down to 5 ng/ml (Figure 2a). Secondary cross-linking
of MCSPxDR5 also increased its agonistic activity toward both melanoma cells (Figure 2b) and primary patient-derived melanoma cells, with a mean increase in apoptosis from 48% to 70% (Figure 2c).

MCSPxDR5 activity was abrogated by cotreatment with recombinant DR5-Fc or pan-caspase inhibitor zVADfmk as illustrated for the melanoma cell line MM-RU (Figure 2d). Moreover, RNA silencing of DR5 in A375M cells strongly reduced the apoptotic activity of both MCSPxDR5 and the DR5-agonistic antibody HGS-ETR2 (Figure 2e and f), whereas treatment with mAb 9.9.27 alone did not induce apoptosis (Figure 2d). Together, these data demonstrate that MCSPxDR5 induces apoptosis through DR5 signaling.

Next, the potential therapeutic effect of Fc cross-linking by surface-expressed Fc receptors was evaluated using HEK293.CD64 cells ectopically expressing the high-affinity Fcγ receptor. HEK293 cells are resistant to TRAIL receptor–mediated apoptosis and lack cytolytic or phagocytic activity (data not shown). In the presence of parental HEK-293 cells and at suboptimal concentrations of MCSPxDR5 (50 ng/ml), only ~25% apoptosis was observed in MM-RU melanoma cells (Figure 2g). However, in the presence of HEK293.CD64 cells, MCSPxDR5 induced apoptosis in up to 60% of MM-RU cells (Figure 2g). Fc receptor–mediated cross-linking of MCSPxDR5-IgG4, an isotype devoid of antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) activity, yielded similar results (Figure 2g). Of note, Fc receptor cross-linking also to some extent induced MCSP-independent DR5 signaling in MCSP-M14 cells (Figure 2g). Nevertheless, Fc receptor–mediated cross-linking on MCSP cells was abrogated in the presence of mAb 9.9.27 (Figure 2h), indicating that MCSP binding of MCSPxDR5 is pivotal for optimal Fc cross-linking. In analogous mixed culture experiments using freshly isolated leukocytes, MCSPxDR5 also induced a dose-dependent decrease in tumor cell viability down to 20% at an effector cell:target cell ratio of 5:1 (Figure 2i). Together, these data indicate that MCSPxDR5 can trigger antitumor antibody-dependent cell-mediated cytotoxic (ADCC) activity in Fc receptor–positive immune effector cells, which may enhance its in vivo efficacy.

In conclusion, we present a DR5 agonist-based bispecific antibody in which high-affinity binding to MCSP leads to melanoma cell-localized activation of DR5. Antitumor activity of MCSPxDR5 was enhanced by Fc receptor–mediated cross-linking by myeloid immune effector cells. This bispecific antibody approach may provide a new avenue to unlock the therapeutic potential of DR5-targeted cancer therapy.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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