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 coapplication 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 coapplications 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.

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

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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

Abbreviations: DR4, death receptor-4; DR5, death receptor-5; MCSP, melanoma-associated chondroitin sulfate proteoglycan; rhTRAIL, recombinant human soluble tumor necrosis factor-related apoptosis-inducing ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

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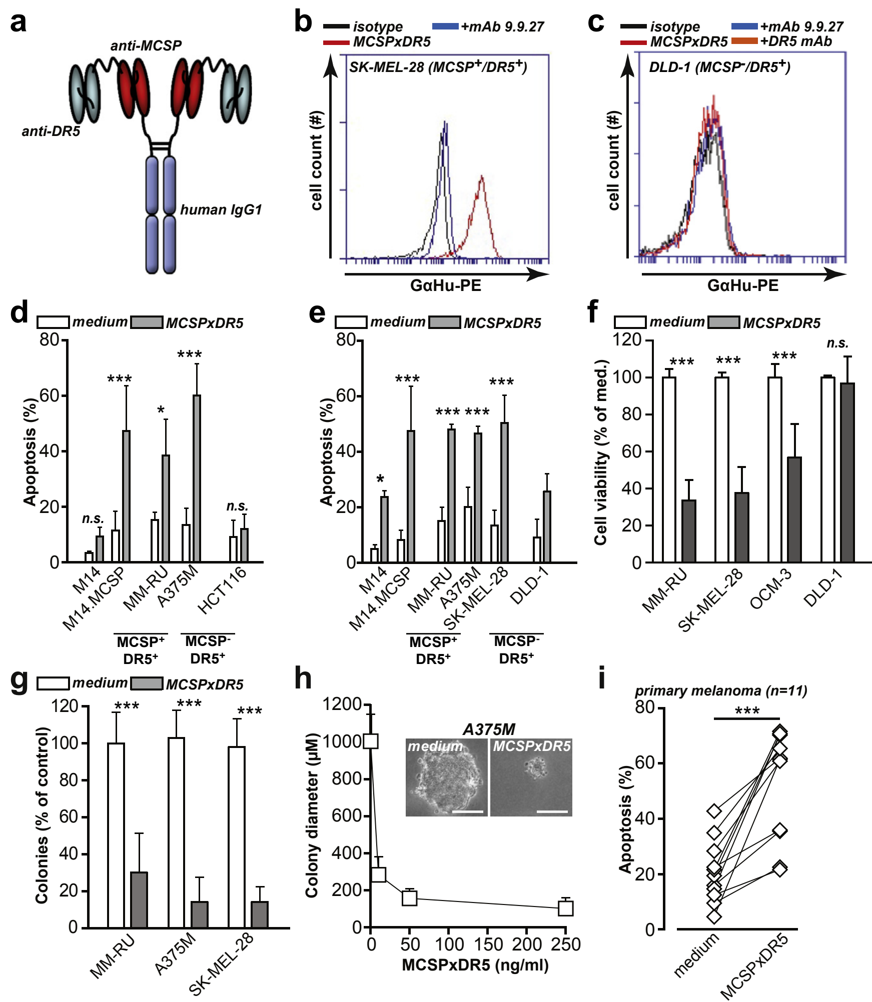

Figure 1. Melanoma-associated chondroitin sulfate proteoglycan (MCSP)-directed apoptotic activity of MCSPxDR5 toward melanoma cells. (**a**) Schematic diagram of the tetravalent bispecific antibody MCSPxDR5. (**b**) SK-MEL-28(MCSP$^+$) and (**c**) DLD1(MCSP$^-$) cancer cells were incubated with MCSPxDR5 (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% CO_2, 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 per condition 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 = 100μm. (**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.

human soluble tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) and agonistic DR4/DR5 antibodies, was well tolerated but had disappointing clinical activity (Ashkenazi, 2015). For instance, in a phase I dose-escalation study in patients with relapsed or refractory carcinoma, the DR5-agonistic antibody tigatuzumab only induced stable disease (Forero-Torres et al., 2010). In a phase II trial in non-small cell lung cancer patients, combined treatment with rhTRAIL and chemotherapy had no added benefit (Soria et al., 2011).

However, advances in our understanding of DR signaling revealed that first-generation DR4/DR5 agonists do not fully exploit the unique signaling characteristics of TRAIL receptor-mediated cancer cell death (Ashkenazi, 2015; de Bruyn et al., 2013). Specifically, rhTRAIL and conventional agonistic DR4/5 antibodies have no tumor-selective binding activity, whereas TRAIL receptors are ubiquitously expressed on normal tissue (Spierings et al., 2004). Consequently, their efficacy may be hampered by a target antigen sink of normal healthy cells. Moreover, DR4 and DR5 have distinct cross-linking requirements for the induction of apoptosis. DR4 is activated upon binding of rhTRAIL (or conventional DR4 antibodies), whereas apoptotic DR signaling requires membrane-bound TRAIL or secondarily cross-linked rhTRAIL (de Bruyn et al., 2010; Nair et al., 2015). Similarly, to gain therapeutic activity, agonistic DR5 antibodies such as tigatuzumab appear to require cross-linking by Fcγ receptors on myeloid effector cells (Bruhns et al., 2009; Li and Ravetch, 2013).

Here, we present a strategy for melanoma-directed activation of apoptosis using a bispecific antibody with specificity for the melanoma-associated chondroitin sulfate proteoglycan (MCSP) and DR5. In brief, a bispecific tetravalent antibody, designated MCSPxDR5, was constructed comprising the binding domains of tigatuzumab and high-affinity anti-MCSP mAb 9.2.27 complemented with a human IgG1 Fc domain (Figure 1a). MCSP is highly overexpressed on the

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