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Necessity is the Mother of Invention: An Ingenious Method for Leukocyte-Targeted Delivery of siRNA in Stabilized Nanoparticles Demonstrates a Role of Cyclin D1 in Inflammation

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Every strategy requires appropriate tools if it is to be feasible. This article describes beautifully designed targeted nanoparticles that can be administered systemically to deliver siRNA for cyclin D1 to specific leukocyte subsets, and further shows that silencing of cyclin D1 in this way can reverse experimentally induced colitis in mice.[1]

Cyclin D1 (CyD1) is a 36-kDa protein that regulates the activity of cyclin-dependent protein kinase in the G1 phase of the cell cycle.[2,3] As breast cancer is protected by CyD1 ablation,[4] CyD1 is of great interest to cancer researchers.[5] However, although CyD1 is strongly expressed at sites of inflammation, its exact role remains unclear. Shimaoka and co-workers chose to attack this problem by using RNA interference to block CyD1 gene expression at sites of inflammation.

A brief comment on RNA interference is called for (Figure 1). Transduction of an artificial double-stranded RNA having complementary base sequences to the targeted mRNA can block gene expression, as demonstrated by Fire et al. in a *C. elegans* model in 1998,[6] work for which A. Z. Fire and C. C. Mello received the Nobel Prize in 2006. The introduced double-stranded RNA is cleaved to 20–23-mer RNA (small interfering RNA, or siRNA) by dicer, an RNase III, and the siRNA is incorporated into an RNA–protein complex called the RNA-induced silencing complex (RISC), leading to cleavage of the targeted mRNA.[7–10] The strategy of introducing an appropriately designed siRNA therefore seems straightforward, but is difficult in practice, because RNA is chemically unstable within an organism,[11] and specific delivery to targeted sites is not easy.[12,13]

The approach used by Shimaoka and co-workers in their study to examine the role of CyD1 in inflammation was to enclose siRNA for CyD1 in stabilized nanoparticles for transport, and to employ suitable antibodies to target β7 integrin (β7I) antigens on the surface of inflammation-associated helper T cells.[14] The authors had previously succeeded in selectively transporting siRNA into leucocytes in vitro and in vivo by using a fusion protein of LFA-1 integrin antibody and protamine.[15] For the present work,[1] stabilized nanoparticles with a covalently attached monoclonal antibody (β7I–tsNPs) were prepared, and siRNA was loaded into them with the aid of protamine.

The structure of β7I–tsNPs is as follows: As cationic lipid polymers are commonly toxic,[16] neutral phospholipids were used to generate unilamellar vesicles of no more than 80 nanometers in diameter. Then, hyaluronan was attached covalently to dipalmitoylphosphatidylethanolamine (DPPE) at the liposome surface to stabilize the particles and to provide a platform for attachment of the antibody.

In order to evaluate the specificity of the obtained tsNPs for target cells, Cy3 fluorescence probe–siRNA (Cy3–siRNA) was loaded into tsNPs bearing a β integrin antibody (β7I–tsNP) or tsNPs bearing an IgG antibody (IgG–tsNP), and incubated with splenocytes expressing β7 integrin or with β7 integrin knock-out (KO) splenocytes. Fluorescence labeling of the splenocytes was examined by flow cytometry. As expected, β7I–tsNP successfully introduced the dye into the wild-type cells. Furthermore, when β7I–tsNP containing siRNA for Ku70, which is expressed ubiquitously in the body, was administered (iv) at the low dose of 2.5 mg kg^{-1} to mice, Ku70 expression in bowel and spleen was inhibited.

For the purpose of this study to examine the role of CyD1 in inflammation, siRNA for CyD1 had to be delivered to the site of inflammation. When ^{3}H-labeled cholesterylhexadecylether was delivered in β7I–tsNP to healthy mice, a substantial portion of the radioactivity was delivered to the gut, whereas little radioactivity was delivered by IgG–tsNP. Furthermore, in mice with dextran sodium sulfate (DSS)-induced colitis, delivery of radioactivity to the gut by β7I–tsNP was about fourfold greater than that in healthy mice.

Because it had been confirmed that β7I–tsNP preferentially targeted leukemic cells at sites of inflammation, the in vitro and in vivo availability of CyD1 siRNA was next evaluated. The in vitro experiment was performed by evaluating CyD1 mRNA with RT-PCR and counting CD3/CD28-stimulated spleen cells. For the in vivo experiment, these items were quantified in bowel and spleen of mice. As had been expected, the expression of CyD1 was decreased only when CyD1 siRNA was delivered in β7I–tsNP. Interestingly, when the relationship between the appearance of cytokines and expression of CyD1 was evaluated, it was found that delivery of CyD1 siRNA in β7I–tsNP suppressed IFN-γ, IL-2, IL-12, and TNF-α, the expression of which is

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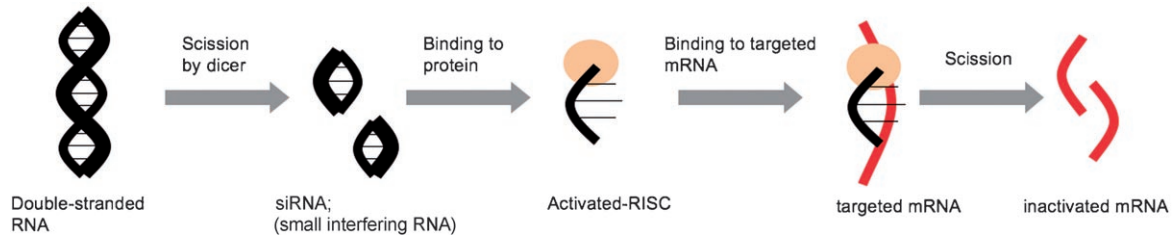

Figure 1. Schematic illustration of RNA interference.

controlled by T helper cells type 1 (T_H1), while expression of IL-4 and IL-10, which are T helper cell type 2 (T_H2) cytokines, was hardly changed. Although the decrease in the amount of T_H1 cytokines by CyD1 knock-out might have been due to cell cycle changes arising from CyD1 suppression, it was established that this was not the case, because the suppression was found to be unrelated to the cell cycle, and was related only to CyD1 knock-out. These results are important evidence that CyD1 itself controls the expression of T_H1 cytokines at sites of inflammation.

Thus, β7I–tsNP containing CyD1 siRNA can control the appearance of inflammatory cytokines at sites of inflammation, and this was confirmed in the DSS-induced mouse model of colitis.[17] Amazingly, DSS-induced colitis mice treated with β7I–tsNP containing CyD1 showed essentially the same weight gain as healthy control mice. Naturally, the untreated DSS-induced colitis mice showed a remarkable weight loss. The therapeutic efficacy in the treated mice was confirmed histologically.

In conclusion, a remarkably effective, systemically administrable, stable nanoparticle delivery system that can transport siRNA selectively to sites of inflammation has been developed. This ingenious tool was used to demonstrate an important role of CyD1 in inflammation, and it is likely to be extremely useful both as a research tool and in developing novel clinical treatments for inflammatory conditions.

Keywords: cyclin D1 · inflammation · nanoparticles · RNA interference · systemic administration

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