Extracts from immature fruit of the apple (Rosaceae, Malus sp.), which contain proanthocyanins (polymers of catechins) as the major ingredients, are known to inhibit histamine release from mast cells. We analyzed in this study the mechanism for the anti-allergic activity of two polyphenol-enriched apple extracts. These extracts, termed “crude apple polyphenol (CAP)” and “apple condensed tannin (ACT)”, reduced the degranulation of mast cells caused by cross-linking of the high-affinity receptor for IgE (FcεRI) with IgE and the antigen in a dose-dependent manner. Furthermore, western blotting revealed that phosphorylation of the intracellular signal-transduction molecules caused by cross-linking of FcεRI was markedly decreased by the addition of CAP or ACT. We then analyzed the effects of CAP and ACT on the binding of the IgE antibody to FcεRI on mast cells, which is the first key step in the allergic reaction mediated by mast cells, and found that this binding was markedly inhibited by both CAP and ACT. These results indicate that the inhibition of binding between FcεRI and IgE by either CAP or ACT was the probable cause of the suppression of mast cell activation. This is the first report demonstrating the molecular mechanism for the anti-allergic effect of proanthocyanin-enriched extracts from apples.

Key words: allergy; apple polyphenol; FcεRI; IgE; proanthocyanin

Apple extracts termed “crude apple polyphenol (CAP)” and “apple condensed tannin (ACT)”, which were prepared from immature apples and contained polyphenols as the main component (over 93%), had an anti-allergic effect on patients with atopic dermatitis and on allergic model mice in vivo. The main polyphenols of these apple extracts are proanthocyanins which are polymers of catechins. Some reports have indicated that polyphenols inhibited the activation of mast cells which are involved in the IgE-mediated allergic reaction. However, the molecular mechanism for the anti-allergic effect of polyphenols is largely unknown. We therefore investigated in this study the effect on mast cells of proanthocyanin-based extracts from apples.

We used in this study bone marrow-derived cultured mast cells (BMMC), because primary cells, unlike cell lines, show a native response to IgE-mediated stimulation. Cross-linking of the high-affinity receptor for IgE (FcεRI) on mast cells by the multivalent antigen via IgE antibodies initiates the activation process, resulting in the release of such chemical mediators as histamine and leukotrienes. To examine the inhibitory effect of ACT and CAP on the degranulation of mast cells, the mast cells were activated by the antigen-IgE complex in the presence or absence of apple extract in a dose-dependent manner (Fig. 1). We thus confirmed that ACT and CAP suppressed mast cell degranulation.

Although ACT and CAP each suppressed mast cell degranulation, the effect of ACT and CAP on the intracellular events in mast cells remained unclear. To
examine the effect of ACT and CAP on the signal-transduction of mast cells, the phosphorylation state of intracellular proteins after IgE-mediated stimulation was analyzed by western blotting, using the 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Many bands reacting with the anti-phosphotyrosine antibody were detected in the activated mast cells (Fig. 2). In contrast, phosphorylation of the intracellular proteins was moderately decreased in the presence of ACT or CAP. When the molecular weights of the detected protein bands were considered, PLC\textsubscript{2}, Syk, Lyn, and LAT could be possible candidates, whose tyrosine phosphorylation through IgE-mediated stimulation was inhibited by ACT and CAP, although these remain to be identified by further study. These observations suggest that signal transduction of the mast cells mediated by FcεRI cross-linking was suppressed by ACT and CAP at an earlier stage of the activation cascade before protein phosphorylation.

All the foregoing observations suggest that the polyphenol-enriched apple extract may have had an anti-allergic effect at an earlier point in allergic signaling. We therefore analyzed the effect of the extracts on binding between the IgE antibody and FcεRI, because binding of the IgE antibody to FcεRI is the first step of IgE-mediated mast cell activation. The flow cytometric technique was used for this examination. In brief, to analyze the amounts of IgE-occupied FcεRI and total FcεRI, the fluorescence of BMMC was detected with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, U.S.A.) after respectively staining with the FITC-conjugated mouse IgE antibody (BD PharMingen, San Diego, CA, U.S.A.) and the FITC-labeled anti-mouse FcεRI \(\alpha\)-chain antibody, MAR-1 (eBioscience, San Diego, CA, U.S.A.), by a previously described method.\textsuperscript{5,6} When BMMC was incubated with the FITC-labeled IgE antibody in the presence of ACT or CAP, the amount of the IgE antibody bound with FcεRI on the mast cells was markedly decreased in a dose-dependent manner by ACT or CAP (Figs. 3Aa, b), whereas the expression level of FcεRI on the mast cells, which was shown by the fluorescence intensity of MAR-1, was not affected by the addition of ACT or CAP (Figs. 3Ad, e). We also examined the effect of other apple-derived low-molecular-weight polyphenols, procyanidins B1, B2 and C1, all of which are presumed to have been contained in the apple extract used in this study, on IgE–FcεRI binding. These polyphenols, however, had no effect on binding between the IgE antibody and FcεRI (data not shown). These results indicate that unidentified compounds, possibly highly polymeric procyanidins, contained in ACT and CAP were responsible for the inhibitory effect on binding between the IgE antibody and FcεRI. A further analysis, using purified polymeric procyanidins, will be required to confirm this, because the involvement of other components contained in the apple extracts cannot be excluded in this study. It is to be noted that Ketotifen, an anti-allergic drug that is often used as a positive control for anti-allergic reactions targeting mast cell degranulation,\textsuperscript{11} also had no significant effect at a non-cytotoxic concentration of 20\(\mu\)g/ml (Fig. 3Ac). This result indicates that the apple extracts inhibited mast cell degranulation by a mechanism different from that of the general anti-allergic drug, Ketotifen. When BMMC that had been pre-incubated with ACT or CAP was reacted with the FITC-labeled

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Inhibition of the Degranulation of Mast Cells in the Presence of Apple Extracts. Various concentrations (1, 10 and 100\(\mu\)g/ml) of CAP and ACT were added to the cell suspension (3 \(\times\) 10\(^5\)) when the cells were incubated with 10\(\mu\)g/ml of the anti-TNP IgE antibody (BD PharMingen) for 1 h at 4 \(^\circ\)C in Tyrode’s buffer. After washing, the cells were stimulated by cross-linking IgE on FcεRI with 30\(\mu\)g/ml of TNP-BSA as antigen (Ag), which had been purified from the reaction mixture of BSA (Sigma-Aldrich) and 2,4,6-trinitrobenzenesulfonic acid sodium salt dihydrate (Wako Pure Chemicals) by PD-10 column chromatography (Amersham Pharmacia). The degree of degranulation was calculated from the \(\beta\)-hexosaminidase activity in the supernatant.\textsuperscript{5} Filled bar, treated with TNP-BSA alone; unfilled bar, stimulated with TNP-BSA after being treated with the anti-TNP IgE antibody.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Phosphorylation State of the Tyrosine Residues in Mast Cells Analyzed by Western Blotting. Whole cells collected after stimulation for 0, 2, 5 or 15 min by IgE and TNP-BSA were applied to SDS–PAGE. The phosphorylated tyrosine residues on the blot were detected by the 4G10 anti-phosphotyrosine antibody.}
\end{figure}
IgE antibody after washing, the amount of IgE antibodies bound to FcβRI was decreased in a dose-dependent manner by ACT or CAP with much lower activity (about 10%) than that for coexistence (Figs. 3Bf, g). This result suggests that the apple extracts inhibited binding between the IgE antibody and FcβRI partly due to suppression of the IgE binding activity of FcβRI. To reveal the possible interaction of ACT or CAP with the IgE antibody itself, a further detailed analysis will be required.

During the preparation of this manuscript, an analysis of the anti-allergic effect targeting mast cells of a catechin-based compound (the O-methylated form of (−)-epigallocatechin-3-O-gallates; EGCGMe) from tea leaves was reported. In that report, degranulation and phosphorylation of several protein tyrosine kinases were shown to be inhibited by the addition of the tea catechin. The authors concluded from those observations that mast cell inhibition could be explained at least in part by the ability of EGCGMe to directly inhibit the function of protein tyrosine kinases. We found in our study decreased phosphorylation of the tyrosine residues of proteins, possibly Lyn and Syk (Fig. 2). Therefore, there is a possibility that the components contained in ACT and CAP might have directly affected the protein phosphorylation as well. The effect of the apple extract and/or completely purified polyphenol on the intracellular events of stimulated mast cells should be analyzed in the near future. This is the first report indicating that the inhibition of binding between IgE and FcβRI, leading to blockage of the phosphorylation cascade, is the most probable primary target for the anti-allergic activities of polyphenol-enriched apple extracts, although the effect of the polyphenol-enriched extracts on mast cells should be analyzed in vivo.

CAP contained procyanidins as the main components (approximately 50%), and procyanidins are even riches in ACT (75–80%). Therefore, the slightly higher activity of ACT in the present study (Figs. 1, 2 and 3) suggests that procyanidins were the main active compounds in the apple polyphenols. Both ACT and CAP inhibited the binding between IgE and FcβRI, whereas procyanidins of low molecular weight (procyanidins B1, B2 and C1), which are dimers (B1 and B2) and a trimer (C1) of

Fig. 3. Effects of the Apple Extracts on Binding between the IgE Antibody and FcβRI.
A, Various concentrations of CAP or ACT and an antibody (the FITC-labeled IgE antibody or FITC-labeled anti-mouse FcβRI α-chain hamster antibody) were added to the cell suspension at the same time after blocking Fc receptors on the cell surface with 2.4G2 (BD Pharmingen). After 1 h of incubation, the cells were washed twice with PBS and analyzed by a FACSCalibur flow cytometer (BD Biosciences). Negative control cells without staining (thin black line), or cells stained with FITC-labeled antibodies in the presence of CAP or ACT at 1 µg/ml (red line), 10 µg/ml (green line) or 100 µg/ml (blue line), or in the absence of apple extracts (thick black line) are shown in Figs. 3a, b, d and e. Negative control cells without staining (thin black line), or cells stained with the FITC-labeled antibody in the presence of Ketotifen at 20 µg/ml (red line) or 40 µg/ml (blue line), or in the absence of Ketotifen (thick black line) are shown in Fig. 3c. The FITC-labeled IgE antibody (Figs. 3a, b and c) or FITC-labeled anti-mouse FcβRI α-chain antibody, MAR-1, (Figs. 3d and e) was used. B, Cells were incubated with CAP (Fig. 3g) or ACT (Fig. 3f) at 1 µg/ml (red line), 10 µg/ml (green line), 100 µg/ml (blue line), or 1 mg/ml (yellow line), or without apple extracts (thick black line) in the culture medium for 1 h. After washing twice with PBS, the cells were stained with the FITC-labeled mouse IgE antibody as already described. Negative control cells without staining are shown by the thin black line. The fluorescence intensity of each sample was monitored by FACSCalibur after washing with PBS.
epicatechins, did not inhibit the binding, suggesting that the highly polymeric procyanidins in ACT and CAP suppressed the binding between IgE and FcεRI. A further study with purified compounds from ACT will be needed to clarify this aspect.

Cross-linking of FcεRI induced by the complex formation of IgE with an antigenic protein is an essential event in the IgE-mediated allergic reaction, so that inhibition of the binding between IgE and FcεRI has been a target for the development of anti-allergic drugs. Indeed, an antibody against the Fc portion of the human IgE antibody, which inhibits the interaction between IgE and FcεRI, has been reported to be a powerful tool for suppressing allergic reactions. Therefore, an antibody against the Fc portion of the IgE antibody might be useful for preventing and treating allergic diseases.

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