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GENISTEIN ENHANCES THE ICAM-MEDIATED ADHESION BY INDUCING THE EXPRESSION OF ICAM-1 AND ITS COUNTER-RECEPTORS

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SUMMARY. Binding of circulating cells to endothelium is mediated by recognition between endothelial adhesion molecules and their counter-receptors. The β_2 integrins are a group of adhesion molecules, mainly expressed on leukocytes, that mediate intercellular binding by recognizing their counterparts on endothelial cells, among others ICAM-1. In this study we have studied the regulation of this interaction in myelomonocytic cells treated with genistein, a tyrosine kinase inhibitor with several other biological functions. We show that genistein upregulates the surface expression of the β_2-integrins in the monoblastic THP-1 and to a lesser extent in the promyelocytic HL-60 leukemia cell lines. This upregulation leads to an increase in the adherence of THP-1 cells to ICAM-1. Genistein also modulates the expression of ICAM-1 on endothelial cells by potentiating the upregulating effect of TNF and IFN-γ. Genistein may thus enhance intercellular binding by affecting both the endothelium and the circulating cells.

The extravasation of circulating cells requires recognition between the adhesion molecules on the surface of the endothelial cells and their counter-receptors on the circulating cells. The circulating cells are initially slowed down by a recognition between selectins and their carbohydrate counterparts, which causes them to roll along the vessel wall. This then allows the recognition between integrin adhesion receptors and their counterparts belonging to the immunoglobulin superfamily (1). The β_2 integrins LFA-1 and Mac-1 bind to intercellular adhesion molecules ICAMs (2-5) whereas the counterpart of the third β_2 integrin gp150,95 is yet uncharacterized (6). In addition to the β_2-integrins, one β_1 integrin, VLA-4 and the two β_7 integrins also participate in intercellular binding via recognition of endothelial adhesion molecules (7-9).

Genistein is an isoflavone compound that has recently drawn attention as a potential anti-cancer drug due to its non-proliferative and differentiating effects on various cancer cells. Among

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ABBREVIATIONS:
ICAM-1, Intercellular adhesion molecule-1; TNF, tumor necrosis factor; IFN-γ, Interferon gamma.

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several other effects, genistein causes differentiation of various myelomonocytic leukemia cell lines (10-14). We have previously shown that PMA differentiated THP-1 cells with an upregulated expression of β_2-integrins have an enhanced capacity to bind to ICAM-1 (15). In this paper we show that treatment of the myelomonocytic HL-60 and THP-1 cell lines with genistein leads to an upregulation of their β_2 -integrin expression. We demonstrate that the genistein-treated THP-1 cells have an enhanced binding-capacity to ICAM-1. In addition, genistein modulates the endothelial ICAM-1 expression by enhancing the upregulation of its surface expression by the cytokines IFN-γ and TNF.

MATERIALS AND METHODS

Reagents. IFN-γ was a generous gifts from C.W.Reynolds, National Cancer Institute (Frederick, MD). TNF was purchased from Boehringer (Mannheim, Germany). The monoclonal antibodies to CD11a (clone IOT16), CD11b (clone IOM1), CD11c (clone IOM11c) and CD18 (clone IOT18) were purchased from Immunotech (Marseille, France). The blocking monoclonal anti CD18 antibody clone 7E4 (16) that was used in adhesion assays was a kind gift from Pekka Nortamo (Helsinki, Finland). The blocking monoclonal anti ICAM-1 antibody UHP-9 was raised as described previously (15). The secondary antibody for flow cytometry was the fluorescein-conjugated F(ab')2 fragment of rabbit immunoglobulins to mouse immunoglobulins purchased from DAKOPATTS a/s (Glostrup, Denmark). ICAM-1 was immunoaffinity purified from placental lysates as described (15).

Cells. HL-60 (17) and THP-1 (18) cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium. The Ea.Hy 926 cell-line (19) was obtained from Cora-Jean Edgell (Chapel Hill, NC) and was cultured in Dulbecco's HAT medium. All media were supplemented with 10% FCS, 10 mM HEPES, 2mM glutamine and antibiotics. For THP-1 cells 70 μM mercaptoethanol was added to the medium. The cells were grown at 37 °C in 5% CO_2.

Flow cytometry. For flow cytometry, the cells were incubated with antibody diluted with washing buffer (0.2%FCS/PBS) at 4 °C for 30 minutes. The cells were then washed twice with washing buffer. The cells were incubated with the fluorescein-labeled secondary antibody as above. After two washings the cells were suspended in 3% PFA/PBS and analysed in the Beckton Dickinson FACSCAN analyser. The results are expressed as the mean fluorescence intensity (MFI).

Adherence assay. Purified ICAM-1 protein was attached to Petri-dishes by incubating a 50 μl dot of protein diluted with 0.2% NaN_3/PBS overnight at 4°C. The spot was marked, the solution was aspirated and the dish was blocked by incubating with 1% BSA/0.2% NaN_3/PBS for at least 1 hr at room temperature or longer at 4°C. The activated and control cells were harvested and suspended to a density of 1-2x10^6 cells/ml in adhesion wash solution 1% FCS/RPMI. The cell suspension was pipetted to the wells and the plates were incubated for 20 minutes at room temperature. For antibody inhibition assay the 2x10^6 cells were incubated with 10 μg/ml of antibody for 30 minutes at +4 °C before they were dispensed to the plates. Non-adherent cells were aspirated and the plates were washed with washing solution until no floating cells were detected. The amount of adhered cells was determined by counting the amount of cells in 10 different microscopic fields at 200x magnification. Non-specific binding was determined by counting cells attached to BSA-fields as above and was substracted from the ICAM-binding score. The average±SEM of three independent assays was calculated.

RESULTS

Genistein increases the surface expression of CD11/CD18 on HL-60 and THP-1 cells. The myelomonocytic cell lines HL-60 and THP-1 were either left untreated or were treated with 3 or 10 μg/ml of genistein for 72 hours. The surface expression of CD11/CD18

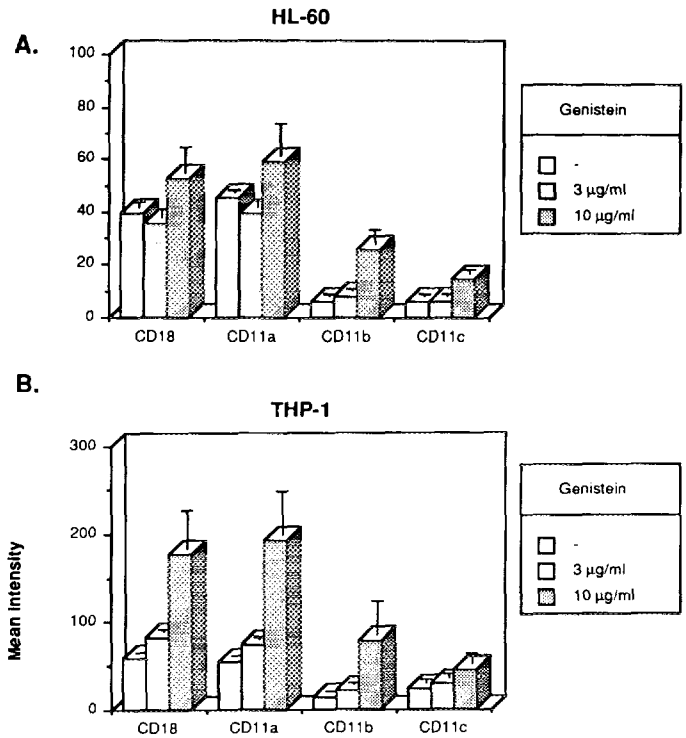

Figure 1. **Genistein upregulates the surface expression of the β_2-integrins.** HL-60 (A) and THP-1 cells (B) were either left untreated (-) or were treated with 3 or 10 µg/ml of genistein for three days. The surface expression of the four β_2-integrin chains was then measured by flow cytometry. The mean intensity ± SE of three independent experiments is depicted.

was then measured by flow cytometry. On HL-60 cells treated with 10 µg/ml of genistein an increase in the surface expression of only CD11b and CD11c was observed (Figure 1A). On the more mature monocytic cell line THP-1 the surface expression of CD18, CD11a and CD11b increased 3-5 fold (Figure 1B). Only a very weak effect was detected when the genistein-concentration was 3 µg/ml, whereas the concentrations of 30 µg/ml and above were cytotoxic. In previous studies we have shown that PMA-differentiation leads to a down-regulation of the surface expression of the VLA-4 α-chain and to the upregulation of the surface expression of the β_7 integrins ((20), Tiisala et al., submitted). These phenomena were not observed in the genistein treated cells (data not shown).

Genistein enhances the increase of ICAM-1 surface expression induced by cytokines. We further tested the possible effect of genistein to the expression of the endothelial adhesion molecule ICAM-1, the counter-receptor of CD11a/CD18 and CD11b/CD18, on the endothelial cell line EA.Hy 926. Genistein alone had only a marginal effect on the surface expression of ICAM-1. It, however, was seen to dose-dependently enhance the upregulating effect of TNF particularly at suboptimal TNF-concentrations. After a 24-hour treatment with

10 ng/ml TNF the mean intensity of ICAM-1 increases over 30-fold (not shown). When the cells were treated with a suboptimal TNF dose of 0.1 ng/ml alone, only a 5-fold increase in the surface expression of ICAM-1 was detected at 24 hours. Simultaneous treatment of the cells with genistein led to a dose-dependent enhancement of ICAM-1 surface expression. Treatment of the cells with 30 μg/ml of genistein together with 0.1 ng/ml TNF led to an 18-fold increase in the surface expression of ICAM-1. The effect on IFN-γ induced surface expression was less pronounced, being approximately two-fold (Figure 2).

The genistein-induced increase in the surface expression of β_2 integrins leads to enhanced cell adhesion. Our previous studies have shown that the quantitative upregulation of the ICAM-counter-receptors leads to an enhanced cell adhesion (15). We therefore tested whether the genistein-treated cells whose CD11/CD18 expression is upregulated have an increased binding capacity to purified ICAM-1. A 2-3 fold increase of the binding of THP-1 cells to purified ICAM-1 was observed when the cells were treated with 10 μg/ml genistein for three days. Short-term stimulation of cells with PMA has been shown to cause a transient increase in their adhesiveness through an increase in the avidity of β_2-integrins. When the genistein-treated cells were further stimulated with 100 nM PMA for 30' the adherence was seen to increase compared to cells treated with genistein alone. The binding could be blocked with the anti-CD18 and anti-ICAM-1 antibodies UHP-9 (15) and 7E4 (16), respectively, whereas the control antibody 84-3C1 was ineffective (Figure 3).

DISCUSSION

In this paper we demonstrate that long-term treatment with genistein of the two myelomonocytic leukemia cell lines, THP-1 and HL-60, upregulates their expression of β_2 integrins. Concomitantly the adherence of the THP-1 cells to ICAM-1 is increased. Genistein also induces the ICAM-1 surface expression of the endothelial cell line EA.Hy926. Thus

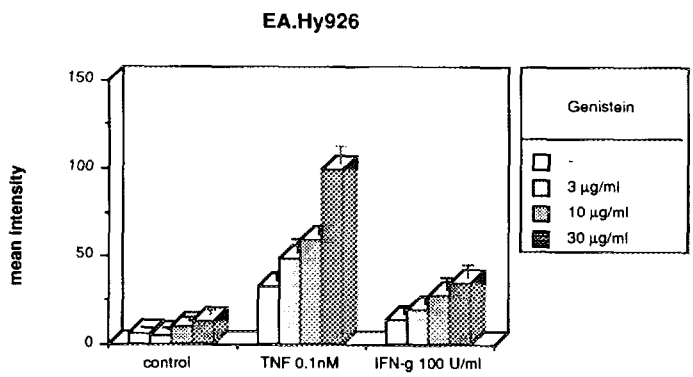

Figure 2. **Genistein enhances the TNF and IFN-γ mediated upregulation of ICAM-1 expression of EA.Hy 926 cells.** The cells were either left untreated (-) or were treated with various concentrations of genistein for 30 minutes before addition of medium alone (control) or 0.1 nM TNF or 100 U/ml of IFN-γ. The surface expression of ICAM-1 was then measured by flow cytometry. The mean intensity ± SE of four independent experiments is depicted.

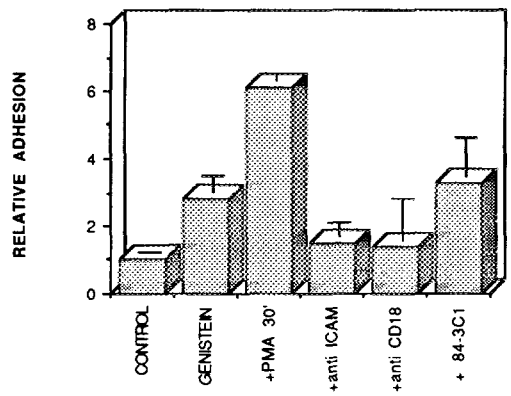

Figure 3. **Increased binding to ICAM-1 of genistein-treated THP-1 cells.** THP-1 cells were left untreated (control) or were treated with 10 µg/ml genistein for three days and the adhesion assay was performed as described in Materials and methods. The binding was blocked with specific antibodies, whereas the control antibody was ineffective. Binding of genistein treated cells could be further increased when the cells were treated with 100 nM of PMA for 30 minutes prior to adhesion. The results are expressed as relative adhesion where the amount of bound cells is scaled to the value of non-treated cells that is given the value of 1. The average ± SE of three independent experiments is depicted.

genistein appears to have the potential to induce intercellular binding by increasing the adhesive properties of both the circulating cells and the endothelium.

Genistein is an isoflavone compound, originally isolated from the fermentation broth of *Pseudomonas* sp. (21). Although originally described as a tyrosine kinase inhibitor (21), genistein has been shown to have several other biological effects. Among them are inhibition of topoisomerase I and II (22), inhibition of phosphatidylinositol turnover (23) and inhibition of histidine kinase (24). Lately, genistein has drawn attention as a potential anti-cancer drug. *In vitro* genistein has been shown to induce differentiation, to inhibit the growth, as well as to cause apoptosis of various cancer cells and also to inhibit angiogenesis (10, 13, 25-32). It has also been suggested that genistein may be more cytotoxic to malignant than normal cells, which increases its attraction as a chemotherapeutic drug (27, 33). The low cancer rate among vegetarians has been hypothesized to correlate to particularly high genistein levels in plasma or urine. For instance the low mortality from prostatic cancer among Japanese men has been suggested to be due to a high plasma isoflavonoid levels, including genistein (34).

An upregulation of the surface expression of β_2 integrins is observed in differentiating myelomonocytic cell lines, among others HL-60 and THP-1 (15, 35). In a previous study we have demonstrated that THP-1 cells with increased β_2 integrin surface expression have an enhanced capacity to bind to purified ICAM-1 compared to non-treated cells (15). The fact that genistein has been reported to induce the differentiation of various murine and human leukemia

cell lines, (10-14) prompted us to investigate the effect of genistein to the integrin expression and binding of THP-1 and HL-60 cells to ICAM-1. The observed changes in the surface expression of the CD11/CD18 chains in THP-1 were very similar to those on PMA-treated cells (15) with the exception of CD11c whose upregulation was rather modest. HL-60 cells are less mature than THP-1 cells and have the potential to differentiate either along the granulocytic, monocytic or macrophage-like pathway (36). In HL-60 cells treated with genistein an up-regulation of only the CD11b chain was observed. This expression pattern is similar to that observed in HL-60 the differentiating along the granulocytic pathway, that may be induced by treatment of with e.g. DMSO or retinoic acid (37). In accordance with these findings, genistein has been reported to induce granulocytic or monocytic rather than macrophage-like differentiation of HL-60 cells (10, 13). Therefore, only THP-1 cells were used in the adhesion assay. Which of the several biological effects of genistein induces the observed upregulation of the surface expression of the β_2 integrins remains unresolved in this study. In studies on the differentiation of HL-60 cells by treatment with genistein, modulation of phosphatidylinositol turnover (13), the inhibition of tyrosine phosphorylation (27) and DNA breakage (10) each in turn have been suggested to be the underlying mechanism of the induction of differentiation.

Also endothelium plays an active role in integrin-mediated intercellular binding by up-regulating the surface expression of adhesion molecules belonging to the immunoglobulin superfamily. This upregulation is induced by cytokines that are released at the site of inflammation, e.g. the expression of ICAM-1 on endothelial cells has been shown to be upregulated by treatment with IFN-γ or TNF. In a recent study genistein was shown to attenuate the adhesion of human leukocytes to endothelial cells and to abrogate the enhancing effect of cytokines to binding to endothelium (38). However, the possible target molecules for the inhibition were unidentified. Also, the endothelial cells were treated with genistein for only 10 minutes, a time that is insufficient to induce a change in the expression level of adhesion molecules. In the present study we provide direct evidence for the ability of genistein to induce the expression of molecules participating in intercellular adhesion. These findings suggest that genistein may enhance the adhesive properties of both the endothelium and the circulating cells. *This effect might be relevant in the therapeutic applications of genistein.*

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