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# Retinoic Acid-Induced Golgi Apparatus Disruption in F2000 Fibroblasts: A Model for Enhanced Intracellular Retrograde Transport

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**Retinoic acid (RA) can transform the Golgi apparatus (GA) into a diffuse vacuolar aggregate and increase the toxicity of some immunotoxins that enter into cells by receptor-mediated endocytosis. An ultramorphological study of the RA-induced GA disruption was performed on F2000 fibroblasts. Cultures were treated with 0.11 to 30 μM RA for 7 - 180 min. The endocytosis of *Limax flavus* agglutinin-peroxidase conjugate (LFA), and the interactions between a phorbol ester (PMA) and RA concerning GA disruption, were examined. Exposure to 0.33 μM RA for 20 min transformed the GA into vacuolar aggregate. These vacuoles were not involved in endocytosis since they remained unstained after endocytosis of LFA. However, the lysosomes were involved in endocytosis, as they were strongly stained. Therefore, a RA-induced shift towards lysosomal routing of the entered LFA was presumed. Exposure to PMA made cells resistant to the Golgi-disturbing effects of RA, indicating that protein kinase C plays an important role in this process.**

**Keywords:** All-*trans* retinoic acid, Endocytosis, Golgi apparatus

## Introduction

All-*trans* retinoic acid (RA) is a polyisoprenoide molecule that plays an important regulatory role in eukaryotic organisms (Allen and Bloxham, 1989; Morré, 1992). Its nuclear receptors (RARs/RXR) bind at regulatory genomic elements. When they are activated by RA, they enhance gene transcription (Morré 1992; Petkovich, 1992; Allenby *et al.*, 1993). RA and its derivatives have long been used in the oncological practice for therapy and prevention of many human neoplastic diseases, such as acute promyelocytic leukemia, squamous cell carcinomas at different sites, basaloid skin cancer in inherited basal cell nevus syndrome, ovarian cancer, neuroblastoma, as well as Müllerian mixed tumors (Lippman, 1997 and references therein). All of these effects are mediated through the RARs/RXR system.

RA is also a Golgi-disturbing agent (Dinter and Berger, 1998) that can transform the Golgi apparatus (GA) into a diffuse vacuolar aggregate. In this case, the toxicity of immunotoxins which enter the cells by receptor-mediated endocytosis can be enhanced 10,000 times (Wu *et al.*, 1994). This effect does not require protein synthesis or RAR activity, and is not due to the increased immunotoxin receptor avidity. Importantly, it can be inhibited by Brefeldin A (Wu *et al.*, 1994), which indicates a possible RA-induced alteration of the intracellular immunotoxin routing through the GA (see also Lippincott-Schwartz *et al.*, 1989; Sandvig *et al.*, 1991). Immunotoxins are promising modern biological response modifiers in anticancer therapy (Jurcic *et al*., 1996; Dillman, 2001). Some are toxic for cellular membranes, while others are targeted at intracellular aims and expound their toxicity after receptor-mediated endocytosis. Enhancement of the latter's toxicity *in vitro* by RA could be of particular importance *in vivo*. Nevertheless, a detailed morphological examination of the RA-induced GA-disruption has not yet been performed. Therefore, it was decided to characterize the morphological and dynamical changes in the intracellular endocytic routes upon RA treatment, as well as to explore the mechanisms of the Golgi-disturbing RA-effects. For these purposes, the tracing of the *Limax flavus* agglutinin-peroxidase conjugate (LFA), which is a substance that enters into cells in a similar way as the receptor-mediated entering immunotoxins, and the effects of phorbol ester on the RA-induced changes in GA, were examined.

## Materials and Methods

**Cell lines and media** Human embryonic pulmonary fibroblasts

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F2000 (Flow Laboratories, Irvine, Scotland) were grown as monolayers for 48 h under standard culture conditions (humidified atmosphere with 5% $CO_2$ at 37°C) on 13 mm round coverslips in one part Dulbecco's modified minimal essential medium of Eagle (DMEM) and one part HAM's F12 medium that contained 10% fetal calf serum and 4 mM glutamine (all from Sigma, St. Louis, USA). The cells reached a density of $2 \times 10^4/cm^2$.

**Retinoic acid** RA (Sigma, St. Louis, USA) was dissolved in 1 ml dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) and stored at –20°C (0.167 M stock solution). Working solutions were prepared by diluting the stock solution in DMEM.

**Phorbolester** Phorbol-12-myristate-13-acetate (PMA, Sigma) was dissolved in DMSO and stored at –20°C (1 µM stock solution). The working solution was prepared by diluting the stock solution in DMEM to 20 nM.

**Tracing of the endocytic routes with LFA-peroxidase conjugate** Peroxidase-labeled LFA (EY-Lab., San Mateo, USA) was dissolved in 0.1 M phosphate buffer saline (PBS) and stored at –20°C (1 mg/ml stock solution). It was further diluted in DMEM/F12 up to a final concentration of 0.3 mg/ml. Cell cultures were incubated with this solution for 1 or 2 h, then in DMEM for 60 min and further processed according to different protocols.

**Electron microscopy** The cell cultures were fixed with 2.5% glutaraldehyde at 4°C and further processed for electron microscopy (post-fixation with 1% $OsO_4$ for 4 h, alcohol dehydration, Epon embedding, ultramicrotomy, uranyl-acetate, and lead citrate staining). The LFA-treated cells were stained with diaminobenzidine (DAB, Sigma) for 15 min (10 mg DAB in 20 ml 0.2 M Tris buffer with 10 µl $H_2O_2$), then post-fixed with osmium ferrocyanide for 15 min, and processed for electron microscopy as described.

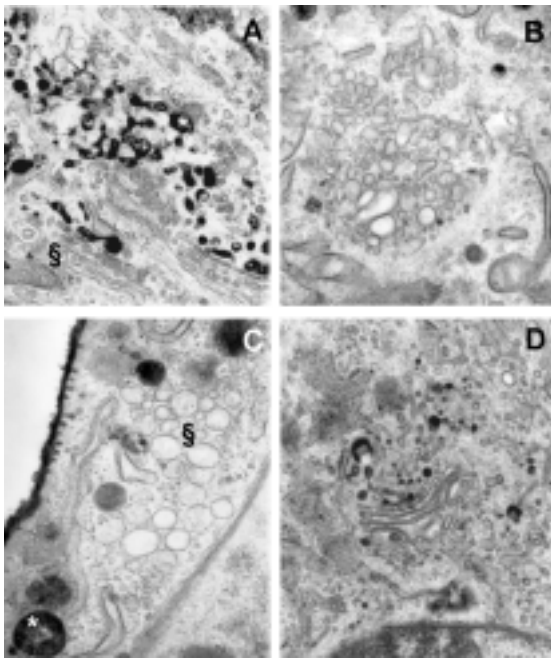


**Fig. 1.** (A) The normal Golgi region in F2000 cells upon endocytosis of *Limax flavus* agglutinin-peroxidase conjugate (LFA). Endocytic compartments as *trans*-Golgi network (*) are stained, while *cis*-Golgi stacks (§) remain unstained. Cultures were treated with LFA for 60 min. (B) Total vacuolization of the Golgi region in F2000 cells after treatment with 0.3 µM retinoic acid for 20 min. Compare to a normal Golgi region in 1A. (C) Endocytosis of LFA in F2000 cells after treatment with retinoic acid. Plasmalemma, and especially lysosomes (*), are strongly stained; the vacuolated Golgi region remains unstained (§). Cultures were simultaneously treated with 10 µM retinoic acid and LFA for 60 min. (D) Reconstitution of the LFA-routing in F2000 cells after retinoic acid removal. Cultures were treated with 10 µM retinoic acid for 60 min, washed, incubated with the medium for 60 min, and then with LFA for 60 min.

## Results

**RA-induced GA disruption, RA concentration, and incubation time** The F2000 cells were treated with 0.33, 1, 3, 10, and 30 µM RA for 7, 20, 60, 120, and 180 min. The GA of the medium and DMSO control cultures remained unchanged (a normal Golgi region after tracing of LFA is shown in Fig. 1A). Treatment with 0.33 µM RA for 7 min caused a subtle GA vacuolization and displacement of the Golgi complex towards the plasmalemma. A total vacuolization of the GA occurred in the cultures that were treated with 0.33 µM RA for 20 min (Fig. 1B). Prolonging the incubation time or increasing the RA concentration induced no additional morphologic alterations.

**Tracing of endocytic routes under the influence of RA** The F2000 cells were either simultaneously or subsequently incubated with LFA and 10 µM RA for 60 min. The medium and DMSO control cultures showed neither morphologic alteration, nor changed LFA-routing through the endocytic compartments: plasmalemma, multivesicular bodies, mitochondrial membranes, *trans*-Golgi network and the *trans*-most cisterns were stained, while the *cis*-Golgi stacks remained unstained (Fig. 1A). Plasmalemma, multivesicular bodies, and mitochondrial membranes were as strongly stained in the experimental groups as in the controls, while the Golgi complex showed distinct staining-pattern changes (Fig. 1C):

- RA-induced vacuoles remained unstained
- Lysosomes were markedly stained
- Endoplasmatic reticula remained unstained.

**F2000 cells reassemble functionally their GA upon RA removal** The F2000 cells were treated with 10 µM RA for 60 min, then left in DMEM for 60 min and incubated with LFA for an additional 60 to 120 min. All of the cell cultures were capable of reassembling their GA and even restoring their ability to uptake LFA in the *trans*-most Golgi cisterns.

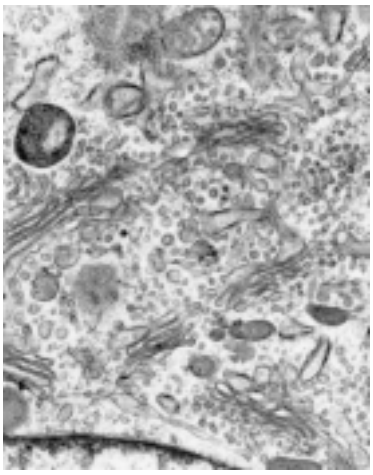

**Fig. 2.** Exposure of F2000 cells to phorbol-12-myristate-13-acetate (PMA) induces Golgi apparatus resistance towards the retinoic acid-induced transformation. Note that there are only stacks and parts from the *trans*-Golgi network, but no retinoic acid-induced vacuoles. The cultures were incubated with PMA for 4 h, then washed and incubated with 10 μM retinoic acid for 60 min.

Therefore, the RA-induced GA disruption appeared to be functionally reversible (Fig. 1D).

**RA-PMA antagonism concerning GA disruption** Three repeats, using the same protocol, showed high reproducibility. The F2000 cells were either simultaneously treated with 10 μM RA and 20 nM PMA for 60 min or with 10 μM RA for 60 min after pre-incubation with 20 nM PMA for 4 h. The controls that were treated with 20 nM PMA for 60 min showed no morphologic changes in their Golgi region. PMA/RA co-incubation weakened slightly the RA-induced GA disruption: some more resistant GA and fewer vacuoles were observed. RA could not evoke any changes in the GA of the cells that were exposed to PMA for 4 h (Fig. 2). Therefore, incubation with PMA made GA resistant towards the RA-induced disruption.

## Discussion

In this study, it was shown that RA can selectively disrupt the GA of F2000 cells. These results correlate with the results of Wu and co-authors (Wu *et al.*, 1994), which showed RA to cause a complete vacuolization of the Golgi complex in human and rat glioma cells. It was further demonstrated that upon RA removal, the F2000 cells can not only reassemble the morphological integrity of their GA, like rat glioma cells (Wu *et al.*, 1994), but even reconstitute their physiological endocytic routes, as assessed by the tracing of LFA. Exposure to RA concentrations of 0.33 μM for 7 to 20 min initialized a GA transformation in the F2000 cells. It was calculated that under these circumstances each cell was exposed to approximately $1 \times 10^8$ RA molecules *in vitro*. Morré (Morré *et al.*, 1988) and Varani (Varani *et al.*, 1996) demonstrated that RA can change the membrane fluidity of membranous organelles. It was supposed that the amount of $1 \times 10^8$ RA molecules (as in our experimental setting) could not exclusively induce such profound morphological changes in the GA, preserving all other intracellular organelles, if "simple" fluidity alteration of the intracellular membranes took place. It was further supposed that the observed-RA effects may occur in an RAR-independent way that involves protein kinases and second messenger cascades (Anderson *et al.*, 1985; Cope and Boutwell, 1985; Cope, 1986; Nowack *et al.*, 1990; Zhao *et al.*, 1990; Evain-Brion *et al.*, 1991; Tang and Ziboh, 1991; Kurie *et al.*, 1993; Bouzinba-Segard *et al.*, 1994; Kahl-Rainer and Marian, 1994; Carter *et al.*, 1998; Morré *et al.*, 1998). The experimental evidence that the RA-induced GA-transformation could be suppressed by exposure to phorbol ester supported this assumption, and clearly demonstrated that some components of the protein kinase C (PKC) cascade play an important role in the RA-induced GA disruption. RA is a partial high affinity diacylglycerol agonist-antagonist, which binds to the diacylglycerol-sensitive allosteric enzyme center of PKC (Cope, 1986; Tang and Ziboh, 1991; Bouzinba-Segard *et al.*, 1994; Kahl-Rainer and Marian, 1994). PKC plays a key regulatory role in the function of many cytoskeletal components and membranous organelles (Toker, 1998). Moreover, some RA-induced rearrangements of intracellular filaments and translocations of enzymes are mediated by signaling pathways that involve components of the PKC cascade (Kurie *et al.*, 1993; Carter *et al.*, 1998).

RA can change the endocytic routing of immunotoxins, thereby, increasing their toxicity (Wu *et al.*, 1994). Therefore, it was of particular interest to explore the endocytic cellular pathways in a dynamic tracing experiment with LFA (Hedman *et al.*, 1986; Pavelka *et al.*, 1998). The RA-induced vacuoles were not involved in endocytosis since they remained unstained after the LFA-uptake. However, the lysosomes appeared to be stained stronger than in the controls, therefore they were involved in endocytosis. The endoplasmatic reticulum (ER), as a possible gate for the endocyted substances into the cytoplasm, remained unstained. Thus, there were no morphologic signs of increased retrograde transport of the endocyted LFA to the disrupted Golgi region or through it into the ER, which indicates that the endocytic GA-capacity decreased or even disappeared after incubation with RA. It is still unclear how the obviously-suppressed intracellular transport routes to GA and ER could explain the RA-enhanced toxicity of immunotoxins that enter into cells by receptor-mediated endocytosis (Wu *et al.*, 1994). An indication for the activation of other intracellular transport routes that leads to increased transport of endocyted substances through the late endocytic compartment could be the strongly LFA-stained lysosomes. Concerning this fact, lysosomal membrane permeabilization that might be caused

by the RA-induced changes of lipid fluidity could play an important role in the penetration of the endocyted LFA and immunotoxins, respectively, into the cytoplasm (Morré *et al.*, 1988; Varani *et al.*, 1996). A rapid penetration through the RA-induced vacuoles could be yet another unproven mechanism of the increased entrance of endocyted substances into the cytoplasm. Future ultrastructural studies should clarify the exact way of immunotoxin penetration in the RA-treated cells.

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