IgG immunoglobulins induce activation of the sphingomyelin cycle in HL-60 cells

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Abstract In HL-60 cells signal transduction via sphingomyelin hydrolysis (sphingomyelin cycle) is induced by binding of tumor necrosis factor α (TNFα) to cell surface TNFα receptor. We found that IgG immunoglobulins activate sphingomyelin hydrolysis in plasma membrane of HL-60 cells, with kinetics similar to that of activation by TNFα. Activation was induced by different IgG isotypes (most of which are irrelevant to known inducers of the sphingomyelin cycle) and also by Fcγ fragments of IgG. The facts that inhibiting the binding of the antibodies to the cell surface by protein A prevents activation of sphingomyelin hydrolysis and that soluble TNF receptor of 55-kDa subtype (TBP_{55}) inhibits activation, suggest that the mechanism of IgG-induced sphingomyelin hydrolysis involves binding of IgGs through their Fcγ domain to Fcγ surface receptors which mediate autocrine secretion of TNFα. The latter is responsible for inducing sphingomyelin hydrolysis. This study suggests that TBP_{55} may be an effective inhibitor of the sphingomyelin cycle.

Key words: Signal transduction; Sphingomyelin cycle; TNFα; TBP_{55}; IgG immunoglobulin; Fcγ

1. Introduction

Recently it has become evident that the sphingomyelin (SPM) pathway plays a major role in signal transduction by regulating major stages in cell development such as cell division, differentiation, and apoptosis ([1,2] and references therein). Ceramide, the lipid product of SPM hydrolysis, is formed due to the activation of neutral [3–5] and/or acidic [1] sphingomyelinases in many cell types by tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), vitamin D_3, or nerve growth factor (NGF) [1,6]. The targets of the agents which initiate the signal are specific membrane receptors such as the 55-kDa TNFα receptor for TNFα [7] or the low-affinity neurotropin receptor ($p75^{NTR}$) for NGF [6]. The ceramide formed can either be modified to other signaling products (ceramide-1-phosphate, sphingosine-1-phosphate, or sphingosine) or affect various functions directly by an as yet unknown mechanism. We found that IgG immunoglobulins, including anti-TNFα IgG, through their Fcγ domains, activate the SPM cycle, and that this process is inhibited by soluble 55-kDa TNF receptor (TBP_{55}).

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Abbreviations: anti-αLTX, purified rabbit anti-α latro-toxin; DCCM-1, defined cell culture medium; IL-1β, interleukin-1β; MEMα, minimum essential medium alpha; NGF, nerve growth factor; PBS, phosphate-buffered saline; PC, phosphatidylcholine; SPM, sphingomyelin; TBP_{55}, TNF-binding protein of 55 kDa; TNFα, tumor necrosis factor α

This study was aimed at clarifying the mechanism by which IgG immunoglobulins activate the sphingomyelin cycle and identifying molecules which, through interaction with the agonist (TNFα), inhibit this cycle.

2. Materials and methods

The basic experimental system used was similar to that of Okazaki et al. [8] and Dressler et al. [9]. HL-60 cells were grown in suspension in MEMα medium (Biological Industries, Bet HaEmek, Israel), enriched with vitamin solution and sodium pyruvate and containing 10% fetal calf serum. The cells were grown at 37°C in 5% CO_2 atmosphere in a 25-ml flask at a maximum concentration of 0.5×10^6 cells/ml in the presence of 7.3×10^5 dpm/ml [^{3}H]choline (15 Ci/mmol, Amersham, Little Chalfont, Buckinghamshire, UK) for 48–72 h. Then the medium was removed and cells were washed (×2) in phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}). The washed cells were incubated in 24-well plates in DCCM-1 medium (without fetal calf serum) (Biological Industries) at a concentration of 0.5×10^6 cells/ml (final volume per well 1 ml) for the desired time (0–130 min) at 37° in 5% CO_2 atmosphere, in the presence of the agents described in the text, table, and figures. Incubation was terminated by cell lipid extraction [10]. The chloroformic enriched lower phase was washed ×2 with synthetic upper phase (chloroform:methanol:water 6:94:96 by volume) and the lower phase was evaporated under N_2. After evaporation the samples were dissolved in chloroform:methanol 2:1 (v/v). Pure egg phosphatidylcholine (PC, Lipoid, Ludwigshafen, Germany) and egg SPM (Avanti Polar Lipids, Alabaster, AL, USA), 22 μg of each, were added to the washed lower phase of each sample as carrier. The chloroformic lower phase was evaporated to dryness, and the residue was dissolved in a small volume of chloroform:methanol 2:1 (v/v) and applied to silica gel G (scored 10×20 cm, 250-μm TLC plates) (Analtech, Newark, DE, USA) [11]. The plates were developed in chloroform:methanol:water 65:35:5 (v/v). More than 90% of the radioactivity in the lipid was found in PC and SPM. For untreated cells at time 0 the labeling ratio [^{3}H]PC/[^{3}H]SPM was in the range of ~4:1. Neither TNFα nor various IgG antibodies induced hydrolysis of PC in the timescale of our experiment; therefore the ratio of [^{3}H]SPM to [^{3}H]PC served as a more accurate measure of the level of SPM hydrolysis since it corrects for losses in extraction. Each experimental point was repeated in triplicate; standard deviation of each point was ≤10%. Based on the trypan blue vital staining test, 15–20% of the HL-60 cells died during the 30-min incubation with 30 nM TNFα, while no cell death occurred due to incubation with the various IgG antibodies used in this study. For more details see legends to Table 1 and figures.

3. Results and discussion

3.1. Anti-TNFα IgG and other unrelated IgGs activate the SPM cycle

It was previously reported that high concentrations of anti-TNFα antibodies inhibited the SPM cycle [12,13]. We tried to inhibit the TNFα-induced SPM hydrolysis activation in HL-60 cells using lower concentrations of human anti-TNFα IgG polyclonal antibodies. Rabbit anti-human TNFα-neutralizing polyclonal antibodies (primarily IgG, Genzyme Cytokine Research Products, Cambridge, MA, USA) and human rTNFα

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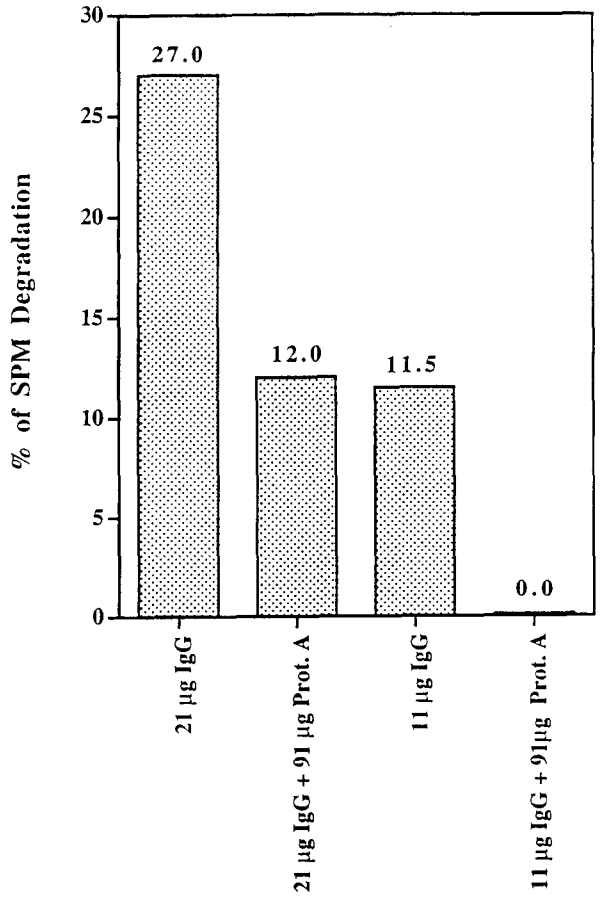

Fig. 1. Inhibition of IgG-induced [^{3}H]sphingomyelin hydrolysis in HL-60 by protein A. [^{3}H]SPM labeled HL-60 cells were incubated with either 21 or 11 μg/ml rabbit anti-αLTX IgG antibodies or the same two concentrations of anti-αLTX which were preincubated with 91 μg/ml of protein A for 45 min. For more details see Section 2.

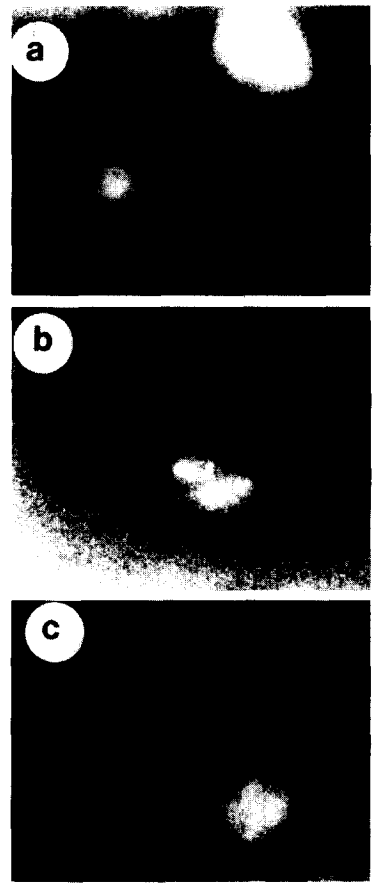

Fig. 2. Involvement of IgG Fcγ domain in IgG-HL-60 cell interaction. a, b, c describe micrographs in which the fluorescence level of anti-rabbit FITC F(ab)$_2$ is distributed on the surface of HL-60 cells (a,b,c in Table 1). For more details see text.

(Chiron, Emmersville, CA, USA) were used in this experiment. To our surprise, not only did the polyclonal IgG anti-TNFα antibodies not inhibit the HL-60 SPM hydrolysis, they actually more than doubled it from 13 ± 2.0% for 30 nM TNFα alone to 27 ± 2.0% for 30 nM TNFα in the presence of 1:50 dilution of anti-TNFα antibodies. In order to assess the role of the anti-TNFα antibodies in the enhancement of SPM degradation, HL-60 cells were incubated with 50 μl (1:100 dilution) of the above anti-TNFα antibodies in the absence of TNFα. After 30 min incubation, 23.0 ± 2.0% degradation of HL-60 [^{3}H]SPM was obtained, suggesting that the anti-TNFα antibodies can act by themselves as inducers of the SPM cycle. This raises the question of specificity or, in particular, which domain of the IgG molecule is responsible for this activation. We addressed this by incubating [^{3}H]choline-labeled HL-60 cells with either (i) 11 μg of purified rabbit anti-α latro-toxin (anti-αLTX) IgG antibodies (these polyclonal antibodies – a gift of Noam Emanuel, Lautenberg Center for General and Tumor Immunology, Hebrew University - Hadassah Medical School – were raised to neu-

Table 1
Involvement of IgG Fcγ domain in IgG interaction with HL-60 cells

Experimental system						Results		
HL-60 cells	Primary antibodies	Secondary anti-rabbit FITC-F(ab)$_2$	Protein A	4°C incubation (+azide)	37°C incubation (−azide)	Binding (4°C+azide)	Capping (37°C −azide)	Internalization (37°C −azide)
+	−	−	−	+		−	−	−
+	−	−	−	−	+	−	−	−
+	−	+	−	+	−	−	−	−
+	+	+	−	+	−	+[a]	−	−
+	+	+	−	−	+	+	+	+[b]
+	+	+	+	+	−	−[c]	−	−
+	+	+	+	−	+	−	−	−

[a,b,c]Micrographs a,b,c in Fig. 2.

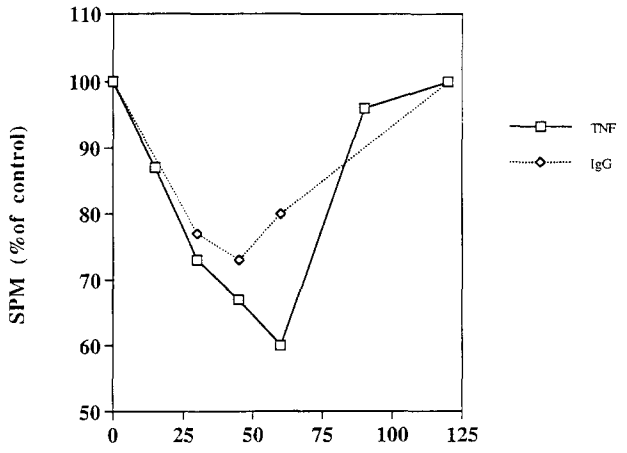

Fig. 3. Effect of TNFα and IgG on the time-dependent levels of [^{3}H]SPM in HL-60 cells. [^{3}H]SPM-labeled HL-60 cells were incubated in the presence of either 30 nM TNFα (□) or anti-αLTX IgG (◇) (50 μg/well). Incubation was carried out as described in the text for the specified time. For more details see Section 2.

tralize spider poison and therefore are irrelevant to TNFα neutralization or binding) or (ii) 50 μg protein of a fraction of unspecific human immunoglobulins which is enriched with IgG (Octagam, Octapharma AG, Glarus, Switzerland).

Both the specific rabbit anti-αLTX antibodies and the human immunoglobulins activate the SPM cycle, reaching 20.0 ± 2.0% SPM degradation. Since the only common denominator of all these three antibody fractions is the Fcγ domain, we studied directly the involvement of this domain (i) by incubating HL-60 cells with purified unreduced 2-chain Fcγ fragment (Jackson Immuno Research, West Grove, PA, USA) and (ii) by studying the effect of protein A (Zymed, South San Francisco, CA, USA) on the SPM hydrolysis induced by IgG antibodies. The unreduced 2-chain Fcγ fragment activates the SPM cycle inducing 23.0 ± 3.0% SPM hydrolysis in a way similar to that of intact antibodies. More support for the primary involvement of the Fcγ domain of IgG molecules comes from the results (Fig. 1) which demonstrate that protein A, which binds with high affinity to the Fcγ domain of most IgGs other than subclass IgG_3 [14], inhibits the IgG-induced SPM hydrolysis in a manner which is dependent on the ratio of protein A to IgG.

3.2. Mechanism of IgG-induced activation of SPM cycle

To study the mode of interaction of IgG antibodies with the HL-60 cells we incubated cells with 10 μg/ml anti-αLTX IgG antibodies in MEMα medium containing 0.5% sodium azide. After 30 min at 4°C, FITC-labeled secondary $F(ab)_2$ derived from goat anti-rabbit antibodies lacking the Fcγ domain (Sigma, St. Louis, MO, USA) were added. The cells were incubated at 4°C for 30 min, washed ×2, and then transferred to azide-free medium at 37°C. For both temperatures (4°C and 37°C) the distribution of the antibodies in the cells and on the cell surface was followed by fluorescence microscopy. The results are described in Table 1 and Fig. 2. The results in Table 1 clearly show that the primary antibodies bind to the cell surface at 4°C in the presence of azide (Fig. 2a), while incubation at 37°C in the absence of azide leads to capping and internalization (Fig. 2b). This binding is inhibited by protein A (Fig. 2c). This experiment also demonstrates that the $F(ab)_2$, which lack the Fcγ domain, do not activate the SPM cycle.

In order to elucidate the involvement of TNFα in the IgG-induced [^{3}H]SPM hydrolysis we first compared the kinetics of hydrolysis induced by TNFα and anti-αLTX IgG. Fig. 3 shows that there is a similarity in the kinetics between the TNFα-induced SPM hydrolysis and antibody-induced hydrolysis. It is well established that the TNFα-induced hydrolysis has a typical kinetic profile. The hydrolysis starts immediately after contact of the cells with TNFα and in HL-60 cells reaches a maximum at about 30–60 min post-contact with TNFα, followed by a recovery phase and reaching the levels of before the interaction of the cells with TNFα (or even higher values) of [^{3}H]SPM at 90–120 min after initial contact with TNFα [5,9]. The profile of the kinetics of hydrolysis induced by anti-αLTX IgG was similar to that obtained with TNFα, except that the level of maximum hydrolysis was higher (Fig. 3) for TNFα-induced SPM hydrolysis. The involvement of TNFα in the process could not be investigated using anti-TNFα antibodies (see above). Therefore we looked for a monovalent agent that has TNFα-binding properties similar to the membrane TNFα receptor which participates in the SPM cycle. TBP_{55}, the stable, single-chain, single-site, 55-kDa TNFα-binding protein (a gift of Prof. D. Wallach, Weizmann Institute of Science, Rehovot, Israel) is such an agent. It inhibits TNFα-induced SPM hydrolysis in a concentration-dependent manner. TBP_{55}/TNFα in a molar ratio of 3.3 (100 nM/30 nM) results in 16% inhibition of [^{3}H]SPM

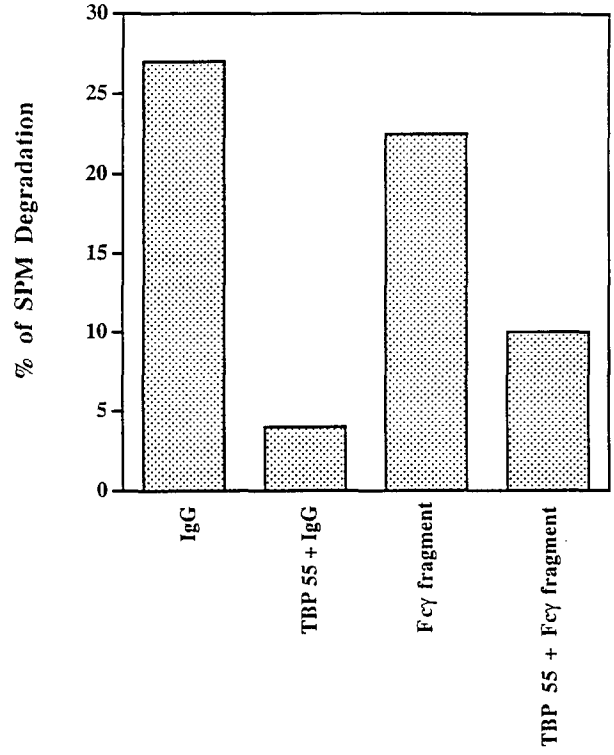

Fig. 4. TBP_{55} inhibits IgG- and Fcγ fragment-induced hydrolysis of [^{3}H]SPM in [^{3}H]SPM-labeled HL-60 cells. [^{3}H]SPM-labeled HL-60 cells (see text) were incubated for 45 min at 37°C in DCCM-1 medium containing either 21 μg rabbit anti-αLTX IgG antibodies (see Fig. 1) or 50 μg Fcγ fragments either in the presence or absence of 540 nM TBP_{55} (30 μg/well) for 40 min at 37°C. For more details see Section 2.

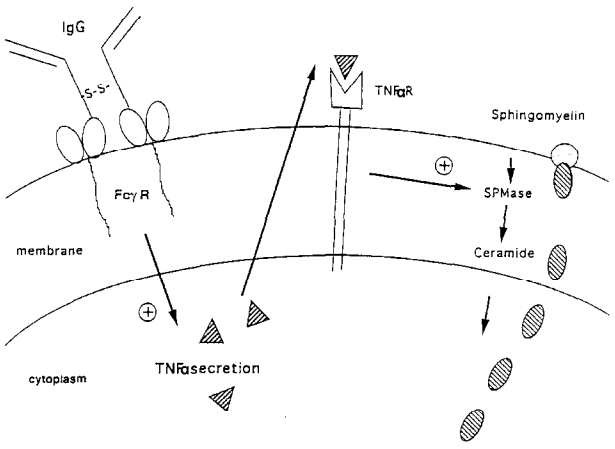

Fig. 5. Scheme of proposed mechanism for IgG-induced sphingomyelin hydrolysis in HL-60 cells. Dimerization (or oligomerization) of FcγR by Fcγ domain of IgG induces fast release of TNFα, which activates the sphingomyelin cycle in the autocrine and/or paracrine modes.

hydrolysis, while in a molar ratio of 30 (900 nM/30 nM), inhibition is 47%. When the TBP_{55} was added to the incubation mixture containing HL-60 cells and anti-αLTX IgG antibodies or containing HL-60 cells and Fcγ fragments, hydrolysis of SPM was either almost completely inhibited (for IgG) or reduced (Fcγ fragments) (Fig. 4).

TBP_{55} inhibition of IgG-induced SPM hydrolysis is greater than TBP_{55} inhibition of SPM hydrolysis induced by 30 nM TNFα. This is explained by the high molar ratio of 1000:1 (TBP_{55}/ TNFα) required to obtain complete neutralization of TNFα [15,16] and the lower TNFα concentration present in the IgG-induced SPM cycle than in the HL-60 cells incubated with 30 nM TNFα. These experiments suggest that TBP_{55} can be used to study if a mechanism of inducing or inhibiting a process is mediated by secreted TNFα. For example, vitamin D_3-induced activation of the SPM cycle in HL-60 cells [8] is also inhibited by TBP_{55}, suggesting mediation by autocrine and/or paracrine secretion of TNFα (Glick and Barenholz, unpublished data).

Studies on the interaction of the IgG antibodies (or Fcγ fragments) with HL-60 cells (Table 1) suggest that the primary event is the binding of the antibodies to the Fcγ receptor on the HL-60 cell surface, which leads to Fcγ receptor (FcγR) cross-linkage, the latter inducing autocrine and/or paracrine TNFα secretion, which activates the SPM cycle (Fig. 5). The hydrolysis of SPM can be inhibited either by preventing binding to cell membrane Fcγ receptors, as exemplified by protein A, or by neutralizing the secreted TNFα by its soluble receptor protein TBP_{55}.

It can be assumed that the cytokine antidotes were secreted as a response to elevated levels of the cytokines. Therefore, one may anticipate that in vivo the kinetics of TNFα secretion and the balance between TNFα and TBPs may control major events mediated by the SPM cycle, such as differentiation and apoptosis of white blood cells. This may explain some of the clinical effects observed during treatment with immunoglobulins [17].

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