Receptor for Advanced Glycation End Products Plays a More Important Role in Cellular Survival than in Neurite Outgrowth during Retinoic Acid-induced Differentiation of Neuroblastoma Cells*

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The receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily, is known to interact with amphoterin. This interaction has been proposed to play a role in neurite outgrowth and process elongation during neurodifferentiation. However, there is as yet no direct evidence of the relevance of this pathway to neurodifferentiation under physiological conditions. In this study we have investigated a possible role of RAGE and amphoterin in the retinoic acid-induced differentiation of neuroblastoma cells. The functional inactivation of RAGE by dominant negative and antisense strategies showed that RAGE is not required for process outgrowth or differentiation, although overexpression of RAGE accelerates the elongation of neuritic processes. Using the antisense strategy, amphoterin was shown to be essential for process outgrowth and differentiation, suggesting that amphoterin may interact with other molecules to exert its effect in this context. Interestingly, the survival of the neuroblastoma cells treated with retinoic acid was partly dependent on the expression of RAGE, and inhibition of RAGE function partially blocked the increase in anti-apoptotic protein Bcl-2 following retinoic acid treatment. Based on these results we propose that a combination therapy using RAGE blockers and retinoic acid may prove as a useful approach for chemotherapy for the treatment of neuroblastoma.

Retinoic acid (RA), a derivative of vitamin A, exerts profound effects on the differentiation, morphogenesis, and survival of many cell types, including neuronal precursor cells (1–3). RA is a natural morphogen that determines anterioposterior axial patterning and induces neuronal differentiation during embryogenesis (4, 5). Human and mouse neuroblastoma cells extend neurites and elongate axons following RA exposure. RA accomplishes most of its biological functions through interaction with two classes of nuclear receptors, the RA receptors (RAR) and retinoid X receptors (RXR) (6–8). Of the naturally occurring retinoids, all-trans-retinoic acid (ATRA) binds to both RAR and RXR, whereas 9-cis-retinoic acid binds to RXR. The signal is then transduced by the formation of RXR/ RAR heterodimers, which bind to RA response elements to activate the transcription of RA-responsive genes.

Retinoic acid-induced differentiation leads ultimately to apoptosis in many cell types indicating close links between the molecular pathways of cell differentiation and those of cell death. Indeed, ATRA reduces the growth of human neuroblastoma cells by inducing differentiation and apoptosis (3, 9, 10) together with growth arrest at the G1 phase of the cell cycle (11, 12). This makes retinoic acid treatment an attractive approach for differentiation therapy of cancers that resist surgery. Clinical trials are in progress to determine the efficacy of retinoids in cancer such as neuroblastoma, which accounts for 15% of cancer deaths in children (13, 14). The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily, which binds to amphoterin, a member of high mobility group protein, to initiate neurite outgrowth in cortical neurons (15, 16). The RAGE-amphoterin interaction elicits Rac and cdc42 activation and stimulates neurodifferentiation in RAGE-expressing cells cultured on amphoterin-coated plates (17). RAGE-amphoterin is localized at the leading edge of differentiating neurons, suggesting a role in axonal and dendritic elongation.

Although retinoic acid-induced neurodifferentiation is mediated by interaction with nuclear receptors, the molecular details of how exactly the nuclear message leads to axonal and dendritic outgrowth at the cell membrane level is still not clearly understood. An improved knowledge of the pathways that link differentiation and apoptosis during retinoic acid-induced differentiation would provide a basis for the development of better therapeutic approaches for neuroblastoma treatment. The present investigation was designed to study the role of RAGE in retinoic acid-induced neurodifferentiation. The mouse neuroblastoma Neuro2a and human SH-SY5Y cell lines chosen as the model for this study extend processes and also undergo apoptosis following retinoic acid treatment. The results presented here show that RAGE-amphoterin interaction plays a critical role in growth retardation and survival of neuroblastoma cells following retinoic acid treatment. The results...
also suggest that RAGE has a supplementary rather than an essential role in process outgrowth during retinoic acid-induced differentiation.

EXPERIMENTAL PROCEDURES

Plasmids and Oligonucleotides—Human RAGE cDNA was a gift from Dr. David Stern, Columbia University, New York. The cytoplasmic deletion mutant of human RAGE was prepared as previously described (14).

Phosphorothioate antisense oligonucleotides were obtained from Sigma-Genosys Ltd., UK. The oligos were 5'-labeled with fluorescein to follow transfection efficiency. The names, sequences, and orientation of the sense and antisense oligos used in this study are presented below in Table I.

Cell Culture and Transfections—The mouse neuroblastoma cell line Neuro2a and the human neuroblastoma cell line SH-SYSY (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) were maintained in DMEM containing heat-inactivated 10% FCS, t-glutamine, penicillin, and streptomycin. Endogenous RAGE expression in both the cell lines was confirmed by Western and Northern analyses.

Construction of Stable Cell Lines—Transfection of Neuro2a cells was carried out with FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Germany) and plasmid at the ratio of 3:1. Stable cell lines expressing full-length RAGE (RAGE-FL), RAGE cytoplasmic deletion mutant (RAGE-Δ), and mock cells harboring the expression vector pcDNA3 were prepared by selection in medium containing 400 μg/ml G418 (Invitrogen). Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane in a semi-dry blotting apparatus (Bio-Rad). The membranes were blocked with 5% nonfat dry milk for all other proteins. The membrane was then incubated with the primary antibody followed by secondary antibody conjugated with alkaline phosphatase. The bands were subsequently detected following the addition of the sensitive coloring agents nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine. Primary antibodies used: Anti-RAGE antibody was a gift from Dr. Neeper (Merck Sharp & Dohme Research Laboratories, Germany). Anti-α-amhpterin antibody and recombinant rat amphoterin were produced and purified as has been described previously (18). Anti-Bcl-2, Bcl-X<sub>L</sub>, and proliferation cell nuclear antigen (PCNA) were purchased from PharMingen. Dr. Larry Denner (Texas Biotechnology Corp.) donated the Anti-sRAGE antibody.

Immunocytochemistry—Cells grown on 2-well chamber slides were fixed with 4% paraformaldehyde for 10 min, washed with PBS, pH 7.4, and permeabilized with 0.1% Triton X-100. The cells were blocked with 5% (w/v) bovine serum albumin (BSA) in PBS for 1 h and incubated with the antibodies anti-RAGE (1 μg/ml) or anti-Bcl-2 (1 μg/ml) overnight at 4°C. Slides were then washed with PBS and mounted with mountant containing 1% p-phenylendiamine (Sigma) prior to analysis using a fluorescence microscope.

Cell Viability and Apoptosis Detection—Cell viability was studied by the MTT assay, and apoptosis was detected by analyses of DNA fragmentation by agarose gel electrophoresis.

MTT Reduction Assay—At the end of each experiment, 10 μl of MTT (5 mg/ml) was added to each well and incubated at 37 °C in 5% air/5% CO<sub>2</sub> for 4–5 h. The insoluble formazan formed was dissolved in isopropanol/0.1 M HCl, and the absorbance was measured in a spectrophotometer at 570 nm with a background reading of 660 nm. The percentage of cell survival was calculated relative to control (taken as 100%).

Agarose Gel for DNA Fragmentation—The detached cells and the adherent cells were processed together for DNA extraction. Detached cells floating in the medium were centrifuged at 500 × g for 10 min and washed with PBS once. Adherent cells were trypsinized, washed with PBS, and pelleted. The pellet was digested in proteinase K and RNase A at 50 °C for overnight. After addition of DNA extraction solution, DNA was obtained by isopropanol and ethanol precipitation. DNA (10 μg) was examined on 1.5% agarose gel, stained with ethidium bromide. DNA size calibration was performed using a 100-bp marker.

RESULTS

RAGE Is Not Required for Retinoic Acid-induced Neurodifferentiation, but RAGE Overexpression Increases the Length of Process Outgrowth

Previous reports (17, 19) on the effect of RAGE on neurite outgrowth in neuroblastoma cells were made using cells that do not express endogenous RAGE. In this study, we chose cells that express endogenous RAGE to study its contribution to cellular differentiation and outgrowth under the influence of a physiological agent, retinoic acid. We constructed stable...
Neuro2a cell lines expressing either full-length RAGE (RAGE-FL) or a cytoplasmic domain deletion mutant of RAGE incapable of signaling (RAGE-Δ) (Fig. 1, A and B). Neuro2a cells treated with ATRA showed sprouting within 12 h, and process outgrowth was clearly observed from 24 to 48 h in agreement with previous findings (20). FITC-phalloidin staining to visualize filamentous actin (Fig. 2A) showed no significant difference in morphology between mock (pcDNA3), RAGE-FL (RAGE full-length), and RAGE-Δ (RAGE cytoplasmic deletion mutant) cells. Compared with untreated control, ATRA treatment for 48 h resulted in 10.0 ± 1.1% of differentiation in mock cells (Fig. 2B). RAGE-Δ cells, which are known to function as a dominant negative mutant for RAGE (21), also extended neuritic processes and were equally well differentiated (10.5 ± 1.1%) as the mock cells (Fig. 2, A (panel c) and B). However, the percentage of differentiation was significantly higher in RAGE-FL cells (15.2 ± 1.7%) (Fig. 2B), that is, the number of cells undergoing differentiation was significantly increased when RAGE is overexpressed. As a complementary approach, inhibition of RAGE expression by antisense oligos (Table I) (Fig. 1C) in Neuro2a cells also failed to show any effect either on the morphology (data not shown) or frequency of differentiation following retinoic acid treatment. After 48 h of differentiation, Neuro2a cells treated with antisense oligos (AS-mRAGE1) showed a frequency of 11.0 ± 1.3% of differentiated cells. It was noted that treatment of Neuro2a cells with antisense oligos (AS-mRAGE) resulted in 50–60% inhibition of RAGE expression (Fig. 1C).

Neurite Outgrowth after 7 Days of Differentiation—Continuing the ATRA treatment for a week resulted in extensive outgrowth and elaborate network of the processes (Fig. 3A), and the percentage of differentiation remained significantly higher in RAGE-FL (20.8 ± 3.1%) compared with mock (15.6 ± 1.0%) or RAGE-Δ cells (14.4 ± 1.4%) (Fig. 3B). In addition, some of the RAGE-FL cells exhibited extensive elongation of bidirectional processes (Fig. 4A). Cells bearing processes more than 10 times the diameter of cell body were present in significantly higher number among RAGE-FL (8.1 ± 2.1%) compared with mock cells (2.9 ± 1.4%) (Fig. 4B), demonstrating that overexpressed RAGE can contribute to the extension of retinoic acid-induced outgrowths even though its absence may not inhibit the initiation of outgrowth.

Survival of Retinoic Acid-treated Cells Is RAGE dependent; RAGE Plays a Role in Bcl-2 Production during Retinoic acid-induced Neurodifferentiation

Although RAGE-Δ cells showed no significant difference in retinoic acid-induced differentiation or neurite outgrowth, compared with the control after 7 days of retinoic acid treatment, we did observe a reduction in the number of cells remaining on the plates (Fig. 3A (panel c)) suggesting that RAGE-Δ cells may detach from the plate or undergo cell death much earlier than mock or RAGE-FL cells. To address the question whether RAGE-Δ cells are susceptible to accelerated growth arrest and/or increased cell death, we analyzed the expression of the proliferation marker PCNA as an index of growth arrest, and MTT reduction assay and DNA fragmentation pattern analyses were carried out to study cell death. Western analysis of the expression of PCNA after 24 and 48 h of ATRA treatment is shown in Fig. 5A. Mock cells showed a mildly reduced expression of PCNA after 24 h and no detectable expression at 48 h. In RAGE-Δ cells, PCNA expression was already strongly reduced within 24 h of ATRA addition, implying that the functional inactivation of RAGE accelerates growth arrest in ATRA-treated cells. RAGE-Δ Cells Undergo Accelerated Cell Death following ATRA Addition—As detected by MTT reduction analyses, after 7 days of ATRA treatment, compared with untreated control cells (taken as 100%), only 11.5 ± 0.7% of RAGE-Δ cells survived against 43.0 ± 1.4% of mock cells (Fig. 5B). Analyses of the DNA fragmentation pattern provided additional evidence for the increased susceptibility of RAGE-Δ cells to retinoic acid-induced apoptosis. Agarose gel electrophoretic analysis of DNA extracted from RAGE-Δ cells revealed laddering from day 3 onward, whereas no clear laddering was noted in DNA extracted from mock and RAGE-FL cells even after 5 days (Fig. 5C). Similarly, inhibition of RAGE expression by antisense oligos AS-mRAGE1 (Table I) also resulted in reduced survival of ATRA-treated Neuro2a cells. Treatment of Neuro2a cells with ATRA in the presence of sense oligos (5-mRAGE1) for 5 days reduced the percentage of survival to 51.0 ± 3.5% of untreated control (taken as 100%) whereas cells treated under similar conditions with ATRA and antisense oligos (AS-mRAGE1) showed only 26.0 ± 2.1% survival. These findings suggest that functional inactivation of RAGE decreases the survival of retinoic acid-treated cells.

RAGE Transmits an Anti-apoptotic Signal during Retinoic Acid-induced Differentiation—The level of expression of anti-apoptotic molecules of the Bcl-2 family proteins is a critical factor known to influence the susceptibility of a cell to undergo apoptosis. Changes in the expression levels of Bcl-2 family proteins have been reported (22–24) during the retinoic acid-induced differentiation of neuroblastoma cells and are likely to contribute to the altered susceptibility of these cells to apoptosis (24). We therefore studied the expression of Bcl-xL/Bcl-2 proteins to examine the molecular mechanism underlying the increased susceptibility of RAGE-Δ cells to apoptosis. Western...
analyses revealed the endogenous expression of Bcl-2 and Bcl-XL in Neuro2a cells (Fig. 6, A–D), and the level of expression of Bcl-2 was increased from 24 h of ATRA treatment in mock cells (Fig. 6B) whereas that of Bcl-XL remained unchanged (Fig. 6A). A similar increase in Bcl-2 expression was noted in RAGE-FL cells (Fig. 6C). However, Bcl-2 expression did not increase in RAGE-Δ cells (Fig. 6C). A densitometric analysis confirmed that, after 36 h of ATRA treatment, mock and RAGE-FL cells had elevated the level of Bcl-2 2.5 ± 0.3- and 2.8 ± 0.26-fold, respectively, relative to the level expressed at 0 h, whereas no elevation was observed in RAGE-Δ cells. These findings imply that RAGE may transmit an anti-apoptotic signal through the increased expression of Bcl-2 during retinoic acid-induced differentiation, thereby influencing the susceptibility of these cells to apoptosis.

Antisense Inhibition of Amphoterin Expression Prevents Neurite Outgrowth and Reduces Survival of Retinoic Acid-treated Neuroblastoma Cells

RAGE-mediated neurite outgrowth is known to be propagated by its interaction with the ligand amphoterin (15–17). Because the down-regulation of RAGE showed no significant effect on neurite outgrowth and differentiation, we then studied the role of amphoterin in this process. An antisense strategy was employed to inhibit the expression of amphoterin. Immunostaining revealed high concentrations of amphoterin in the nucleus (Fig. 7A), indicating its role as a chromatin-associated protein. Treatment of Neuro2a cells with AS-AMP (3.0 μM) (Table I), for 48 h significantly down-regulated amphoterin expression (>70%) as indicated by the reduced intensity of fluorescence (Fig. 7A). The effect of amphoterin inhibition on retinoic acid-induced neurite outgrowth and differentiation was subsequently analyzed. The inhibition of amphoterin expression prevented retinoic acid-induced differentiation and neurite outgrowth, as detected by FITC-phalloidin staining (Fig. 7B). However, AS-AMP-treated cells showed early signs of differentiation (i.e., sprouting), demonstrating that amphoterin inhibition prevents the elongation of neurites rather than the early events. Neuro2a cells treated with sense oligos, such as S-AMP, showed 11.9 ± 1.0% of differentiation, whereas cells treated with antisense oligos showed only 4.9 ± 0.8% (Fig. 7C). This shows that, although RAGE is not essential for retinoic acid-induced neurite outgrowth, its putative ligand amphoterin does play an essential role. It also suggests that the functions

### Table I

| Name   | Sequence                                      | Position on the cDNA |
|--------|-----------------------------------------------|----------------------|
| AS-hRAGE | 5'-AACGTGCTTCCGGCTGC-3'                       | +4 +21 (anti-sense)  |
| S-hRAGE | 5'-GCAGCGGAAACAGCAGTT-3'                      | +4 +21 (sense)       |
| AS-AMP  | 5'-TTCACTCGTACTCCCTC-3'                       | +729 +746 (anti-sense)|
| S-AMP   | 5'-GAGAAGAGAAGATGAA-3'                        | +696 +713 (sense)    |
| AS-mRAGE1 | 5'-ACCCAGGCTCTAGCTG-3'                       | +18 +37 (anti-sense) |
| AS-mRAGE2 | 5'-TCTCTCAGCCTGGTTGT-3'                      | +1100 +1118 (anti-sense)|
| S-mRAGE | 5'-GCAGCCTAGCCTGGGTGCTG-3'                    | +21 +41 (sense)      |

*AMP, amphoterin; RAGE, receptor for advanced glycation end products.*

Fig. 3. RAGE-Δ cells extend neurites and establish an elaborate network of processes as mock cells following 7 days of ATRA treatment. A, phase contrast pictures of Neuro2a cells treated with ATRA (20.0 μM) for 7 days. a, mock cells; b, RAGE-FL cells, the arrow indicates a cell with extensive lengthy process; c, RAGE-Δ cells. B, percentage of differentiation of mock, RAGE-FL, and RAGE-Δ cells treated with ATRA for 7 days (see “Experimental Procedures”).

RAGE and Retinoic Acid-induced Neurodifferentiation

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of amphoterin in neurite outgrowth are not necessarily mediated through RAGE.

Because RAGE-FL cells showed extensive process outgrowth and elongation of neuritic processes, we speculated that retinoic acid might increase the expression or secretion of amphoterin during differentiation. To clarify this, the expression of amphoterin following ATRA addition was monitored by Western analysis. However, we were unable to detect changes in the expression of amphoterin during differentiation. To clarify this, the expression of amphoterin following ATRA addition was monitored by Western analysis. However, we were unable to detect changes in the expression following ATRA treatment (Fig. 7D).

We then addressed the role of amphoterin in cellular survival during retinoic acid-induced differentiation. As observed by the MTT reduction assay, cells treated with sense oligos (S-AMP) showed 48.0 ± 3.2% (of retinoic acid-untreated control) of survival whereas amphoterin inhibition using antisense oligos (AS-AMP) significantly reduced the percentage of survival to 35.5 ± 0.7% (Fig. 8A). This result was further confirmed by DNA fragmentation analyses, wherein characteristic DNA laddering was observed in AS-AMP-treated cells following 5 days of ATRA treatment (Fig. 8B). Consistent with the results obtained from RAGE-Δ cells, antisense inhibition of amphoterin expression also led to early growth arrest of Neuro2a cells following ATRA treatment. Expression of the proliferation marker, PCNA, was almost completely inhibited within 24 h of ATRA addition (Fig. 5A). These results parallel those obtained with the RAGE-Δ cells, suggesting that the effect of RAGE on cellular survival during retinoic acid-induced differentiation is achieved through its interaction with amphoterin.

Both dominant negative inhibition of RAGE and antisense inhibition of amphoterin resulted in significant reduction in the survival of Neuro2a cells following ATRA treatment. However, there is a discrepancy between the absolute values obtained from MTT reduction analyses of the individual experiments. Treatment with ATRA for 7 days resulted in more than 90% inhibition of cellular survival of RAGE-Δ cells, whereas under similar conditions, antisense inhibition of amphoterin resulted in only 65% inhibition, compared with untreated control cells. Such a difference in percentage of survival is unexpected if RAGE and amphoterin function in the same pathway through their interaction. One possible explanation could be the difference in the strategy employed to inhibit the function of the respected molecules. Antisense oligo-mediated inhibition of amphoterin expression may not reach a maximum level whereas the dominant negative strategy used in RAGE-Δ cells could exert a maximum inhibition of RAGE function, as reported earlier (21).

**Involve RAGE in Retinoic Acid-induced Differentiation and Survival in Neuro2a Cells Is Reproduced in SH-SY5Y Cells**

Having studied the role of RAGE in the murine Neuro2a cells, we wanted to establish whether or not the function of RAGE in cellular survival during retinoic acid-induced differentiation is also observed in the human neuroblastoma cell line. To this end, we chose the human neuroblastoma cell line SH-SY5Y, which is well established as a model for retinoic acid-induced differentiation and survival during retinoic acid-induced differentiation studies (25, 26). An antisense strategy was employed to block the expression of RAGE. As shown in Fig. 9A inclusion of phosphorothioate oligonucleotides, AS-hRAGE (3 and 5 μM), in the medium for 48 h resulted in 50–70% inhibition of the expression of RAGE in SH-SY5Y cells (Fig. 9A). ATRA treatment in the presence of sense oligos, S-hRAGE, for 7 days showed extensive differentiation of SH-SY5Y cells, as judged by shrinkage of cell body and extension of neuritic processes (Fig. 9B). Consistent with the results obtained with Neuro2a cells, inhibition of RAGE expression by AS-hRAGE oligos showed no effect on differentiation, as judged from the morphology and neurite extension of differentiated cells (Fig. 9B). The notion that RAGE plays a role in cellular survival during retinoic acid-induced differentiation received additional support from the cellular survival experiments conducted on SH-SY5Y cells. Treatment of SH-SY5Y cells with ATRA for 7 days reduced the number of surviving control cells to 46.0 ± 2.8% of an untreated control. Under similar conditions, inhibition of RAGE by antisense oligos reduced survival to 16.7 ± 0.7% (Fig. 9D). The observation was further supported by the analysis of expression of Bcl-2: Inhibition of
RAGE reduced the extent of Bcl-2 induction in ATRA-treated SH-SY5Y cells (Fig. 9C).

**RAGE Blocking Antibodies Reduce Growth of Neuroblastoma Cells Treated with Retinoic Acid**

Having established the role of RAGE in cellular survival during retinoic acid-induced differentiation of neuroblastoma cells, we then asked if the RAGE blocking antibodies, raised against the soluble RAGE (sRAGE) could in principle serve as an agent, together with ATRA, for combination therapy to treat neuroblastoma tumors. Taguchi et al. (21) have recently demonstrated, using a mouse model, that RAGE blocking antibodies prevent tumor growth and metastasis by inhibiting RAGE-amphoterin signaling. Therefore, we hypothesize that a combination therapy using retinoic acid and RAGE blockers may exert the double effect of early cell death and prevention of metastasis.

Inclusion of anti-sRAGE antibodies at a concentration of 50 μg/ml together with ATRA significantly reduced the cellular survival of Neuro2a as determined by MTT reduction analysis. Treatment of Neuro2a cells with ATRA alone for 4 days reduced the percentage of survival to 52.0 ± 5.6% of untreated control cells (taken to be 100%). The presence of anti-sRAGE antibodies under similar conditions significantly reduced the percentage of survival to 33.0 ± 4.2% (Fig. 10A). A similar effect was observed in the RAGE-overexpressing RAGE-FL cells: The percentage of survival was reduced to 37.0 ± 4.2% in these cells. Similar results were obtained with the human neuroblastoma cells SH-SY5Y. However, these cells required a higher concentration of antibody to demonstrate the effect.
FIG. 6. RAGE inactivation suppresses increased expression of bel2 in ATRA-treated Neuro2a cells. A, Western blot analyses of Bel-Xl expression in mock cells treated with ATRA (20.0 μM). Western blot analyses of bel2 expression in mock (B), RAGE-FL (C), and RAGE-Δ (D) cells treated with ATRA (20.0 μM).

FIG. 7. Antisense inhibition of amphoterin expression prevented ATRA-induced process outgrowth and differentiation of Neuro2a cells. A, immunocytochemical analysis of amphoterin expression in Neuro2a after transfection with phosphorothioate oligos S-AMP or AS-AMP. Neuro2a cells were treated with 3.0 μg/liter sense oligos S-AMP or antisense oligos AS-AMP, after 48 h the cells were fixed and immunostained with anti-amphoterin antibodies. a, S-AMP-treated cells; b, AS-AMP-treated cells. B, antisense inhibition of amphoterin expression prevented retinoic acid-induced neurite outgrowth. Neuro2a cells were pre-treated with S-AMP or AS-AMP for 24 h, and ATRA (20.0 μM) was then added together with the respective oligos. After 48 h the cells were fixed and stained with FITC-phalloidin to study the differentiation morphology. a, S-AMP-treated cells; b, AS-AMP-treated cells. C, percentage of differentiation of Neuro2a cells treated with 3.0 μg/liter sense oligos S-AMP or antisense oligos AS-AMP and ATRA (20.0 μM) for 48 h. D, retinoic acid has no effect on the synthesis/expression of amphoterin during differentiation. Neuro2a cells were treated with ATRA (20.0 μM), and cell lysates were analyzed for amphoterin expression. Recombinant rat amphoterin was used as a positive control.

It is widely accepted that the nuclear receptors for retinoic acid, RAR and RXR, mediate most of the initiating events in retinoic acid-induced neurodifferentiation (8, 29). Yet, the various molecular mechanisms that translate the nuclear message to axonal and dendritic outgrowth are poorly understood. Data presented here show that amphoterin, which has been shown to localize to the leading edge of a migrating cell (30), is essential for retinoic acid-induced process outgrowth. The blockage of amphoterin expression by antisense oligos significantly prevented process outgrowth. However, functional inactivation of RAGE, the putative receptor for amphoterin, either by dominant negative or antisense strategies, failed to prevent retinoic acid-induced neurite outgrowth, raising the possibility that amphoterin may interact with one or more other molecules to exert its effect on outgrowth. Indeed, previous reports have shown that amphoterin does in fact interact with other mole-

Treatmet with ATRA for 8 days reduced the percentage of survival to 55.0 ± 2.1% of untreated control cells whereas the inclusion of 100 μg/ml anti-sRAGE antibodies reduced the percentage of survival to 43.0 ± 2.1% (Fig. 10B). The difference in the required concentration of antibody may be due to the difference in the time period required for the cell lines to undergo differentiation, because Neuro2a cells undergo differentiation within 48 h of ATRA treatment, whereas the SH-SY5Y cells took more than a week.

DISCUSSION

Some recent reports (15, 17) have suggested that the interaction between RAGE and amphoterin plays a prominent role in neuritc process extension and outgrowth in neuroblastoma cells and/or neurons. It should be noted that these observations were made by culturing RAGE-expressing cells on amphoterin-coated plates and hence establishing a direct contact between RAGE and amphoterin in vitro. The role of RAGE-amphoterin interaction in neurite outgrowth or axonal elongation induced by physiological differentiation inducers, such as retinoic acid, nerve growth factor, and others, remains unknown. Such experiments on the role of RAGE-amphoterin interaction in neurite outgrowth under the influence of physiological agents are necessary to assess the contribution of RAGE in neural development. A detailed understanding of the role of RAGE-amphoterin interaction in neurite outgrowth under the influence of physiological regulators would provide profound knowledge on the molecular biology of neuronal differentiation. Therefore, the purpose of this investigation was to study the role of RAGE and amphoterin in retinoic acid-induced neurodifferentiation, a process widely believed to play an essential role during early neural development (3–5, 27, 28). To achieve this, we have used dominant negative and antisense strategies to functionally inactivate RAGE and an antisense strategy to suppress amphoterin expression in Neuro2a cells. The effect of RAGE was further confirmed by antisense strategy in human neuroblastoma SH-SY5Y cells.
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provide an additive effect on axonal elongation, provided its
extensive outgrowth of neurites also suggests that RAGE may
rather than the initiation phase of neurite growth.
ment, it is possible that RAGE participates in the process
the other hand, because the overexpression of RAGE dramati-
production, and the subsequent messages may be transmitted to a
be controlled by trkB activation followed by G protein activa-
cells by establishing an autocrine loop involving brain-derived
shown that ATRA induces differentiation of neuroblastoma

molecules, including syndecan (31) and sulfoglycolipids (32),
although the relevance of these interactions in neurite outgrowth
remains uncertain. The possibility of amphoterin interacting
with an as yet unidentified molecule may not be ruled out. The
observation made in this study, that blockage of amphoterin
expression prevented only neurite elongation without any effect
on early sprouting, indicates that the early signals of
retinoic acid-induced differentiation are controlled by mecha-
nisms not linked to amphoterin. Middlemas et al. (33) have
shown that ATRA induces differentiation of neuroblastoma
cells by establishing an autocrine loop involving brain-derived
neurotrophic factor and trkB. Early sprouting of neurites may
be controlled by trkB activation followed by G protein activa-
and the subsequent messages may be transmitted to a
pathway that requires amphoterin for neurite outgrowth. On
the other hand, because the overexpression of RAGE dramati-
cally increased the length of neurites after retinoic acid treat-
ment, it is possible that RAGE participates in the process
outgrowth but the role of RAGE might be in the elongation
rather than the initiation phase of neurite growth.
The observation that the overexpression of RAGE stimulates
extensive outgrowth of neurites also suggests that RAGE may
provide an additive effect on axonal elongation, provided its
expression is increased. RAGE is known to be able to increase
its own expression through an NFκB-dependent positive feed-
back mechanism (34). Data on in vivo expression of RAGE
during early neural development supports this hypothesis. It
has been shown that neurons undergoing differentiation and
functional maturity during early development express signifi-
cantly higher levels of RAGE and amphoterin than do adult
neurons (15, 35). Therefore an increased expression of these
molecules during the early development could possibly contrib-
ute to retinoic acid-induced neurodifferentiation in vivo.
The most interesting observation made in this study is the
role of RAGE-amphoterin interaction in the survival of retinoic
acid-treated neuroblastoma cells, which may have implications
for chemotherapy, because differentiation therapy by retinoic
acid treatment is one of the widely tested current modes of
cancer treatment (13). The data presented here show that
RAGE-amphoterin interaction plays a positive regulatory role
in the survival of retinoic acid-treated neuroblastoma cells.
Inhibition of RAGE resulted in early growth arrest and cell
death in both Neuro2a and SH-SY5Y cells. Because the expres-
sion levels of anti-apoptotic molecules have been shown to
increase during differentiation of neuroblastoma cells (22–24),
we hypothesized that RAGE-amphoterin interaction may
transmit signals necessary for the increased expression of anti-
apoptotic molecules. Recently, Huttunen et al. (19) have shown
that RAGE-amphoterin interaction in N18 neuroblastoma cells
favors cell survival through the expression of Bcl-2. Consistent
with this we observed that RAGE blockage in cells resulted in
failure to substantially increase expression of Bcl-2 in retinoic
acid-treated neuroblastoma cells. These observations suggest
that RAGE-amphoterin interaction contributes to the survival
of cells undergoing retinoic acid-induced neurodifferentiation.

If RAGE and/or amphoterin contribute to Bcl-2 expression
during retinoic acid-induced differentiation, what could be the
possible mechanism? Signal transduction pathways involving
protein kinase C (PKC) activation have been implicated in
Bcl-2 production during differentiation (24, 36). Modulators of
PKC activation have been shown to alter the expression levels
of Bcl-2 family proteins and thus modulate susceptibility to
apoptosis (24). In SH-SY5Y cells, the enhanced expression of
Bcl-2 after retinoic acid treatment was blocked by PKC inhibi-
tors, staurosporine or calphostin C (36). In addition, PKC
activation is also reported to result in phosphorylation of Bcl-2
in myeloid leukemia cells thus rendering the cells resistant to
apoptosis (37). The available literature suggests a prominent
role for PKC in the expression and phosphorylation of Bcl-2
protein. Against this background, it is interesting to note that
Melloni et al. (38) have shown that an amphoterin-like protein
activates PKC-α in murine erythroleukemia cells. A recent
report (39) showed that PKC inhibitors block RAGE-mediated
transendothelial migration of monocytes upon ligation with
β-amyloid, another ligand for RAGE, suggesting that RAGE
activation may activate PKC. These reports suggest a possible
involvement of RAGE or amphoterin activation in PKC activa-
tion, however, additional experimental evidence will be re-
quired to clarify whether or not the PKC pathway is involved in
the regulation of Bcl-2 family proteins by RAGE-amphoterin
interaction.

One interesting question that arises from the present study
is whether the signal that passes through RAGE during reti-
noic acid-induced differentiation is involved in cellular growth
arrest or cell death. The observation that inactivation of RAGE
results in reduced PCNA expression and appearance of DNA
laddering clearly suggests that RAGE is involved in both path-
ways. It is widely known that the molecular mechanisms in-
volved in growth arrest and apoptosis are closely linked. How-
ever, the modulatory roles of retinoic acid in these pathways
remain to be elucidated. Further studies in this area would
provide insight into signaling pathways that are propagated
through RAGE during retinoic acid-induced growth arrest and
apoptosis.
The increased expression of Bcl-2 family proteins during differentiation reduces the susceptibility of neuroblastoma cells to apoptosis (24, 40). On the other hand, the antisense inhibition of Bcl-2 expression induces retinoic acid-induced cell death in human neural precursor cells (41). Therefore, improved knowledge of the mechanisms that inhibit increased expression of anti-apoptotic proteins following retinoic acid treatment would provide better therapeutic strategies for neuroblastoma, which accounts for 15% of cancer-related deaths in children (14). Although the presently available chemotherapy for neuroblastoma is partly successful, the majority of the patients subsequently relapse. Alternative therapeutic approaches using retinoic acid and other agents like histone deacetylase inhibitors as combination therapy are being studied (42). Based on our present results we hypothesized that a combination of retinoic acid and RAGE blockers may substantially increase cell death in neuroblastoma or tumor cells, a combination therapy of retinoic acid and RAGE blockers may be a useful approach for the treatment of neuroblastoma.

In conclusion, the results presented in this work suggest that RAGE is not required for retinoic acid-induced neurite outgrowth but plays a significant role in the elongation of neurites and cellular survival during the process of differentiation. However, amphoterin, a known ligand of RAGE, is essential for both neurite outgrowth and cellular survival, raising the possibility that amphoterin might interact with other molecules to exert its effect on neurite outgrowth. Thus there is a disassociation between the effects of RAGE and amphoterin upon retinoic acid-induced neurite outgrowth but not in cellular survival in Neuro2a cells. Based on these results it is proposed that blocking the function of RAGE together with retinoic acid treatment may be used as a therapeutic approach for early growth arrest and cell death in neuroblastoma.
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Receptor for Advanced Glycation End Products Plays a More Important Role in Cellular Survival than in Neurite Outgrowth during Retinoic Acid-induced Differentiation of Neuroblastoma Cells

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