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Expression of Sialidase Neu2 in Leukemic K562 Cells Induces Apoptosis by Impairing Bcr-Abl/Src Kinases Signaling*

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Chronic myeloid leukemia is a hematopoietic stem cell cancer, originated by the perpetually "switched on" activity of the tyrosine kinase Bcr-Abl, leading to uncontrolled proliferation and insensitivity to apoptotic stimuli. The genetic phenotype of myeloid leukemic K562 cells includes the suppression of cytosolic sialidase Neu2. Neu2 transfection in K562 cells induced a marked decrease (−30% and −80%) of the mRNA of the anti-apoptotic factors Bcl-XL and Bcl-2, respectively, and an almost total disappearance of Bcl-2 protein. In addition, gene expression and activity of Bcr-Abl underwent a 35% diminution, together with a marked decrease of Bcr-Abl-dependent Src and Lyn kinase activity. Thus, the antiapoptotic axis Bcr-Abl, Src, and Lyn, which stimulates the formation of Bcl-XL and Bcl-2, was remarkably weakened. The ultimate consequences of these modifications were an increased susceptibility to apoptosis of K562 cells and a marked reduction of their proliferation rate. The molecular link between Neu2 activity and Bcr-Abl signaling pathway may rely on the desialylation of some cytosolic glycoproteins. In fact, three cytosolic glycoproteins, in the range 45–66 kDa, showed a 50–70% decrease of their sialic acid content upon Neu2 expression, supporting their possible role as modulators of the Bcr-Abl complex.

The understanding of signals and pathways that regulate cell proliferation and apoptosis is crucial in searching devices capable to control diseases such as cancer. In fact, a raised threshold for apoptosis represents a central step in tumorigenesis, and tumors that possess alterations of the cell death signaling system are often resistant to chemotherapy (1, 2). The evasion of apoptosis in response to death signals is achieved by malignant cells using sets of mutations that inactivate pro-apoptotic proteins or up-regulate anti-apoptotic proteins (3).

Chronic myeloid leukemia is a hematopoietic stem cell cancer cytologically characterized by the Philadelphia chromosome, which results from a reciprocal translocation between chromosome 9 and chromosome 22 (4, 5). This mutation leads to the formation of the chimeric fusion protein Bcr/Abl, which manifests an uncontrolled tyrosine kinase activity. The expression of Bcr/Abl also contributes to confer apoptosis resistance after growth factors withdrawal or DNA damage. The ability to escape the normal death program provides a huge proliferative advantage and represents, especially during the accelerated or blast phase of the disease, an obstacle to the successful treatment of this pathology, even when employing the new chemotherapic agent Imatinib (6–10). In fact, Bcr-Abl kinase stimulates a variety of downstream survival pathways, including the mitogen-activated protein kinase cascade, Akt, Src kinases, the signal transducers and activators of transcription, and the nuclear factor κB. Activation of these pathways in Bcr-Abl-positive cells results in an increased expression of several anti-apoptotic proteins, such as Bcl-XL and Bcl-2, which block the release of cytochrome *c* from mytochondria, resulting in inactivation of caspases (11, 12).

In the context of cancer disease and signaling, ever-increasing attention is being focused not only on mutations involving proto-oncogenes and tumor suppressor genes but also on alterations affecting glycosylation of lipids and proteins, such as Myc and p53 (13–15). In fact, to regulate communication with each other and intracellular signaling, normal cells use the tool to attach specific sugars to lipids and proteins. Conversely, the ability to alter many glycosylation processes is instrumental to cancer cells to bypass the regular checkpoints that control cell life and death (16).

The modification of the sialic acid content of glycoproteins and glycolipids is among the most frequently recognized glycosylation aberrations used by cancer cells to assure proliferation (13, 15). It is known that sialic acid-containing glycosphingolipids, such as gangliosides, actively participate in several signal transduction pathways through interaction with tyrosine kinases associated to growth factor receptors (17). Moreover, several sphingolipids such as ceramide and lactosyl-ceramide are protagonists in the apoptotic event (17, 18).

Moreover, some sialoglycoproteins were recognized to play important roles in the modulation of cell survival (13). For example, some mucins, such as MUC4, have been identified as apoptosis suppressors and important regulators of cellular growth (13). It is noteworthy that several integrins were also demonstrated to be deeply compromised in their signaling function by an aberrant sialylation profile (15).

Of course, these findings point to the importance of the enzymes implicated in sialic acid metabolism, including them

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in the complicate system that controls cell survival and proliferation. Among these enzymes, sialidases attracted a particular interest (19). Sialidases or neuraminidases (EC 3.2.1.18) are capable of hydrolyzing sialic acid from different sialoglycoconjugates and are present in mammals as four isoenzymes, characterized by distinct genes, molecular features, and subcellular localization: Neu1, Neu2, Neu3, and Neu4 (20, 21). Alterations in the expression of all sialidases, with the exception of Neu4, still not fully characterized (22), were found to occur in several tumors (23–26). In particular, the cytosolic sialidase Neu2 was unexpressed or repressed in the more aggressive tumors, in correlation with their invasiveness (27, 28). In fact, when Neu2 was introduced by transfection in murine melanoma B16-BL6 cells or in mouse colon adenocarcinoma cells, a strong reduction of the metastatic potential of these cells was observed, because of a decrease in ganglioside GM3, a substrate of Neu2 (27, 28).

Although a significant amount of data has been published on sialidase involvement in solid tumors (19), to the best of our knowledge, the contribution of these enzymes in the pathogenesis of blood cancers such as leukemia has not yet been investigated. We recently reported that sialidase Neu2 is not normally expressed in K562 chronic myeloid leukemic cells (29). On these bases, we decided to transfect these cells with Neu2 cDNA and to examine the changes of the biochemical machinery in relationship with the proliferation potential of the same cells.

We demonstrated that Neu2 expression in K562 leukemic cells leads to impair Bcr-Abl activity, consequently modifying the signaling cascades stimulated by this oncoprotein. These modifications have marked effects on the apoptotic machinery of K562 cells, inducing a decrease of Bcl-2 and Bcl-XL genes expression and, therefore, making the cells more sensitive to apoptotic stimuli. The interaction between Neu2 and these signaling pathways seems to be mediated by the desialylation of specific glycoproteins, promoted by the enzyme.

These findings concur to elucidate the importance of glycoproteins and sialidases in the complex mechanism of apoptosis resistance triggered by Bcr-Abl, with possible implications also on the development of new therapeutic strategies against leukemic cells.

EXPERIMENTAL PROCEDURES

Materials—4-Methylumbellyferyl-*N*-acetyl-D-neuraminic acid (4-MU-NeuAc)[2] and 4-methylumbellyferone, pepstatin A, aprotinin, leupeptin, human fetuin, RPMI 1640 medium, fetal bovine serum (FBS), glutamine, penicillin, streptomycin, Trypan Blue, and Hoechst 33342 were provided by Sigma. The RNeasy mini kit was provided by Qiagen (Milan, Italy), Improm II Reverse Transcription System by Promega (Promega Corporation, Madison, WI), iQ SYBR Green Supermix by Bio-Rad, PVDF membrane, SuperSignal West Pico Chemiluminescent substrate, Coomassie protein assay reagent by Pierce, digoxigenin glycan differentiation kit by Roche Applied Science, and pcDNA3.1 vector, DMRIE-C reagent, and geneticin by Invitrogen. Rabbit anti-human Neu2 was prepared by Prof. E. Monti (30).

Sphingosine was prepared from cerebroside (31) and [1-^{3}H]sphingosine (radiochemical purity over 98%; specific radioactivity, 2.08 Ci/mmol) was prepared according to Zhang *et al.* (32). Ganglioside GD1a was purified from the total ganglioside mixture extracted and purified from bovine brain (33). Radioactive GD1a containing *erythro* C18-sphingosine, isotopically tritium labeled at position 3 ([3-^{3}H(*Sph18*)]GD1a), was prepared according to Sonnino *et al.* (34) and purified by reversed phase high pressure liquid chromatography (homogeneity over 99%; specific radioactivity, 1.2 Ci/mmol) (35). [^{3}H]Thymidine was supplied by PerkinElmer Life Sciences.

Cell Culture and Transfection of HsNeu2 into K562 Cells—The human chronic myeloid leukemic cell line, K562, was purchased from ECACC (European Collection of Cell Culture; Sigma) and cultured in RPMI 1640 supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

cDNAs encoding human Neu2 (*Homo sapiens* Neu2, HsNeu2) and an activity-defective Neu2, characterized by an inactivating mutation in which Asp^{46} (pertaining to the active site of the enzyme) was substituted by Ala, were subcloned into the pcDNA3.1 mammalian expression vector. Then 2×10^6 K562 cells were transfected with pcDNA3.1-Neu2, pcDNA3.1-Neu2(Ala $\rightarrow$ Asp), or pcDNA3.1 using the DMRIE-C reagent, according to manufacturer's guidelines. Stable transfectants were isolated after selection with 700 μg/ml geneticin.

RNA Extraction and Real Time RT-PCR—Total RNA was isolated using the RNeasy mini kit, following the protocol suggested by the manufacturer. Then 1 μg of RNA was reverse-transcribed employing the Improm II reverse transcription system, using random examers.

cDNA representing 10 ng of total RNA was used as template for real time PCR, performed using the iCycler thermal cycler (Bio-Rad). PCR mixture included 0.2 μM primers, 50 mM KCl, 20 mM Tris/HCl, pH 8.4, 0.8 mM dNTPs, 0.7U iTaq DNA polymerase, 3 mM $MgCl_2$, and SYBR Green (iQ SYBR Green Supermix), in a final volume of 20 μl. Amplification and real time data acquisition were performed using the following cycle conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 10 s at 95 °C and 30 s at 57 °C. The fold change in expression of the different genes in Neu2 expressing cells compared with control cells was normalized to the expression of β-actin RNA and was calculated by the equation $2^{-\Delta\Delta Ct}$. The primers used for PCR are reported in Table 1. All of the reactions were performed in triplicate. The accuracy was monitored by the analysis of melting curves.

Sialidase Activity Assay—Mock, Neu2, and Neu2 (Ala $\rightarrow$ Asp) transfected K562 cells were harvested by centrifugation and resuspended in PBS containing 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin. The cells were lysated by sonication and centrifuged at 800 $\times$ *g* for 10 min to eliminate unbroken cells and nuclear components. The crude extract was, subsequently, centrifuged at 200,000 $\times$ *g* for 20 min on

[2] The abbreviations used are: 4-MU-NeuAc, 4-methylumbellyferyl-*N*-acetyl-neuraminic acid; FBS, fetal bovine serum; PVDF, polyvinylidene difluoride; RT, reverse transcription; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.

TABLE 1
Primers used for gene expression

Gene	Forward primer	Reverse primer
Neu2	5′-AGAAGGATGAGCACGCAGA-3′	5′-GGATGGCAATGAAGAAGAGG-3′
Bcl-2	5′-GAGGATTGTGGCCTTCTTTG-3′	5′-CCCAGCCTCCGTTATCCT-3′
Bcl-XL	5′-ACATCCCAGCTCCACATCAC-3′	5′-CGATCCGACTCACCAATACC-3′
Bad	5′-GTTCCAGATCCCAGAGTTTG-3′	5′-CCTCCATGATGGCTGCTG-3′
Bcr-Abl	5′-GGAGCTGCAGATGCTGACCACC-3′	5′-TCAGACCCTGAGGCTCAAAGTC-3′
Src	5′-CAATGCAGAGAACCCGAGAG-3′	5′-TCAGATGAAATTCTTTGGAGAG-3′
Lyn	5′-CCTATACGAAATTGTCACCTATGG-3′	5′-ACGCTCTGTAAGTAGTCAAACG-3′
β-Actin	5′-CGACAGGATGCAGAAGGAG-3′	5′-ACATCTGCTGGAAGGTGGA-3′

TL100 Ultracentrifuge (Beckman) to obtain a cytosolic fraction and a particulate fraction. Aliquots of the supernatant were used for Neu2 sialidase activity determination. Enzymatic activity was assayed using [^{3}H]GD1a, 4-MU-NeuAc, and fetuin as substrates, at pH 5.6 or 7.0, according to Tringali *et al.* (29, 36). One unit of sialidase activity is defined as the amount of enzyme liberating 1 μmol product/min.

Confocal Immunofluorescence Microscopy—3×10^4 Neu2 transfected K562 cells in a volume of 200 μl of medium were centrifuged onto glass slides in a Shandon cytospin centrifuge, briefly air-dried, and fixed with 3% paraformaldehyde in PBS for 30 min. After three washes with PBS, the cells were incubated for 15 min in 50 mM NH_4Cl in PBS and permeabilized with 0.5% saponin in PBS. The cells were then incubated for 1 h with rabbit anti-human Neu2 (1:500) antiserum in 0.5% saponin in PBS and, after extensive washes, with secondary donkey anti-rabbit-Cy2-conjugated antibodies (Jackson Laboratories). The samples were then washed with PBS, and a glass coverslip was mounted with DakoCytomation fluorescent mounting medium (DakoCytomation, Glostrup, Denmark). Confocal laser analysis was performed with the Bio-Rad MRC-1024 system, and the acquired images were processed with Adobe Photoshop software.

Proliferation Assays—For proliferation assay, 5×10^4 mock, Neu2, and Neu2 (Ala → Asp) transfected K562 cells were seeded in 24-well culture plates, and viable cells were counted every 24 h with Trypan Blue for up to 5 days. To assess thymidine incorporation, 1.2×10^6 cells were suspended in RPMI 1640 plus 10% FBS, 24 h before the assay. Then 0.5 μCi/ml [^{3}H]thymidine were added to each well, and after 3 h at 37 °C, the radioactivity incorporated was determined in trichloroacetic insoluble material.

Concerning the soft agar colony formation assay, 0.72% bactoagar was melt in 2 ml of RPMI 1640 plus 10% FBS to constitute the hard agar layer and was spread into 35-mm dishes. 1.5×10^5 mock, Neu2, and Neu2 (Ala → Asp) transfected K562 cells were rapidly suspended in 2 ml of RPMI 1640 containing 10% FBS and 0.36% bactoagar at 37 °C and were distributed onto the hard agar layer. Then the plates were incubated at 37 °C for 2 weeks, and the cell colonies were counted (23).

Apoptosis Sensitivity Assays—The resistance of mock, Neu2, and Neu2 (Ala → Asp) transfected K562 cells to apoptotic stimuli was evaluated culturing cells under serum depletion conditions, for 72h. For Hoechst 33342 staining, 5×10^5 cells were washed twice with PBS and incubated for 15 min with 10 μg/ml Hoechst 33342 at room temperature, in the dark. The cells were then observed using an inverted fluorescence microscope (IX50 Olympus) and photographed.

To detect caspase 3 activation, 1×10^6cells were harvested and lysed by sonication. The activation was followed using an antibody recognizing the cleavage fragment of caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA) (24).

For cytochrome *c* release detection (37), 2×10^6 cells were resuspended in 50 mM PIPES/NaOH, pH 7.4, containing 250 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors. After 30 min of incubation in ice, the cells were homogenized with a glass Dounce homogenizer. Cell lysates were centrifuged at 800 × *g* for 10 min, and the supernatants were subsequently centrifuged at 200,000 × *g* for 20 min. Supernatants containing cytosolic proteins were separated by electrophoresis, blotted to PVDF membrane, and probed with cytochrome *c* antibody (Sigma).

Metabolic Labeling of Cell Sphingolipids with [1-^{3}H]Sphingosine—[1-^{3}H]Sphingosine, kept in methanol, was dried under a nitrogen stream and dissolved in RPMI 1640 medium containing 10% FBS to obtain a final concentration of 3×10^{-8} M (corresponding to 0.4 μCi). 2×10^6 mock, Neu2, and Neu2 (Ala → Asp) transfected K562 cells were incubated in this medium for a 2-h pulse followed by a 24-h chase. The sphingolipid pattern was determined according to Papini *et al.* (38).

Glycoprotein Analysis—30 μg of proteins derived from the membrane and cytosolic fractions of mock, Neu2, and Neu2 (Ala → Asp) transfected K562 cells were separated on 10% SDS-PAGE and transferred onto a PVDF membrane. Sialoglycoproteins were visualized employing the digoxigenin glycan differentiation kit, in accordance with the manufacturer's protocol. α2–6-Linked sialic acid was identified using the *Sambucus nigra* agglutinin lectin, whereas α2–3-linked sialic acid was recognized with the *Maackia amurensis* agglutinin lectin.

Western Blot—25 μg of total, cytosolic, or membrane proteins were separated on 12% SDS-PAGE and subsequently transferred onto PVDF membrane. β-Tubulin was used as internal control to ensure equal loading and transfer of proteins. The following antibodies were used for the assays: anti-β-tubulin, anti-Bcl-2 (Sigma), anti-phospho-Bcr (Tyr^{177}), anti-phospho-Src family (Tyr^{416}) (Cell Signaling, Denvers, MA), anti-Tyr(P), anti-Lyn, anti-Src (Santa Cruz Biotechnology), and anti-Neu2 (30). The densitometric analysis of blots was performed using Quantity One Software (Bio-Rad).

Statistical Analyses—The values are presented as the means ± S.D. Statistical analyses were made using unpaired Student's *t* test. Significance was attributed at the 95% level of confidence ($p < 0.05$).

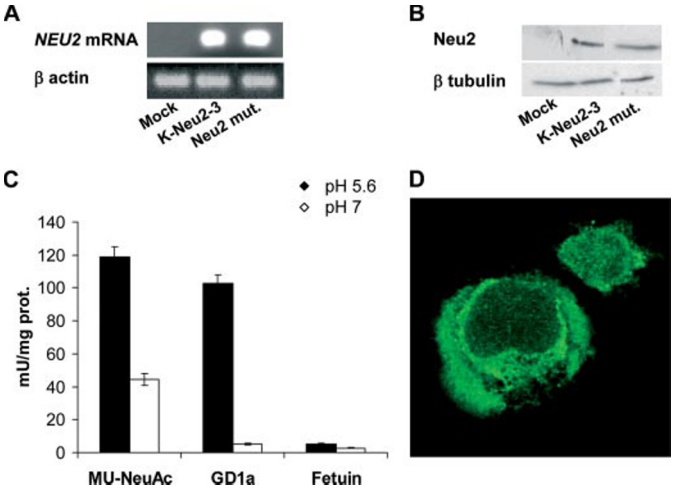

FIGURE 1. **Neu2 expression in K562 cells.** *A*, RT-PCR analysis from total RNA extracted from K-Neu2-3 cells, mock, and Neu2 mutant transfected cells, normalized to β-actin levels. *B*, Western blot of total cell lysates probed with antibody against Neu2. *C*, sialidase activity of the cellular cytosolic fraction obtained by ultracentrifugation at 200,000 × *g* of the total lysate of K-Neu2-3 cells, toward 4-MU-NeuAc, [^{3}H]GD1a, and fetuin, at pH 5.6 (*filled bars*) and 7.0 (*open bars*). The data are the means ± S.D. of five different experiments. *D*, immunofluorescence detection of Neu2 in K562 cells. Specimens were analyzed using a MRC-1024 confocal laser system (Bio-Rad), and the images were processed with Adobe Photoshop software. Original magnification, ×1200.

RESULTS

Neu2 Expression in K562 Cells—In a previous work (29), we demonstrated the absence of endogenous Neu2 sialidase in K562 cells. These findings supported published evidence indicating that many aggressive tumors do not express cytosolic sialidase activity (27, 28). On this basis, we decided to explore the biochemical phenotype of K562 cells with regard to malignancy on the same cells transfected with Neu2 cDNA.

Following the transfection protocol described in the experimental section, we yielded the isolation of eight stable clones, which were checked for Neu2 mRNA expression by RT-PCR, Neu2 protein by Western blot with anti-Neu2 antibody, and Neu2 catalytic activity by enzymatic assay on the cytosolic fraction (200,000 × *g* supernatant). We selected for our study the clone named K-Neu2-3, which showed the highest enzymatic activity.

As controls, we used stable mock transfectants carrying the pcDNA 3.1 vector alone and a stable clone of K562 cells transfected with Neu2 (Ala → Asp), a mutant form of Neu2 in which Asp^{46}, one of the amino acids characteristic of Neu2 active site (39), was substituted by Ala. The mutated protein was proved to lack completely enzymatic activity.

As shown in Fig. 1 (*A* and *B*), K-Neu2-3 and K562 cells transfected with Neu2 (Ala → Asp) mutant showed the presence of both the corresponding mRNA and Neu2 as protein. The expression level of Neu2 and mutant Neu2 in the clones was similar, as emerged by the quantification of the respective mRNA and protein content, by RT-PCR and Western blot, respectively (Fig. 1, *A* and *B*).

The Neu2 sialidase expressed by the K-Neu2-3 clone displayed (Fig. 1*C*) a broad range of substrate recognition, from artificial substrates such as MU-NeuAc (119 ± 10.2 milliunits/mg protein), to gangliosides such as GD1a (103 ± 6.3 milliunits/mg protein), and α2–3 sialoglycoproteins, such as fetuin (5.2 ± 0.4 milliunits/mg protein), with an optimal pH of 5.6 in all cases. Interestingly, Neu2 showed a conspicuous activity toward all the three substrates, also at pH 7.0 (44.6 ± 2.7 milliunits/mg protein with MU-NeuAc, 5.1 ± 0.4 milliunits/mg protein with GD1a, 2.8 ± 0.06 milliunits/mg protein with fetuin), supporting the notion that the living cells acquired an efficient cytosolic sialidase activity. As expected, both mock and mutant transfectants did not present any sialidase activity under the same experimental conditions.

To define the cell localization of expressed Neu2, K-Neu2-3 cells were subjected to confocal microscopy analysis. Fig. 1*D* shows an internal spread staining in a confocal plane of the cells expressing Neu2, clearly indicating a cytosolic distribution of the enzyme.

Control experiments showed that the expression of the other sialidases, Neu1 and Neu3, naturally present in K562 cells, was not affected by Neu2-induced expression (data not shown).

Neu2 Reduces the Cell Proliferation Rate—Aimed at recognizing the effects of Neu2 activity inside leukemic cells, we first checked the cells growth potential. The investigation was carried out at three different levels: by a growth curve obtained counting viable cells every 24 h, by a measurement of [^{3}H]thymidine incorporation, and by a colony formation assay in soft agar.

In all of the assays, the K-Neu2-3 cells revealed an evident impaired proliferation capacity. Growth curve experiments evidenced that after 5 days of culture, the viable cells were significantly less (−55%), as compared with mock cells (Fig. 2*A*). This important decrease of growth rate may be directly connected to a slowed progression through the cell cycle as shown by a 30% reduction of [^{3}H]thymidine incorporation into DNA of the K-Neu2-3 cells (Fig. 2*B*). Importantly, transfectant cells carrying the activity-defective form of Neu2 showed a proliferation rate and [^{3}H]thymidine uptake practically equal to those of mock cells, suggesting that the reduction of cell proliferation is directly linked to the enzymatic activity of Neu2. This trend was confirmed by the colony formation assay in soft agar because K-Neu2-3 cells produced fewer colonies than mock and Neu2 mutant transfected cells and either died or remained as single cells for up to 2 weeks in culture (Fig. 2*C*).

Neu2 Partially Restores the Susceptibility to Apoptotic Stimuli—Neu2 expressing K562 became less resistant to apoptosis than control cells (Fig. 3). In fact, when cells were cultured for 3 days under serum deprivation to stimulate apoptosis, K-Neu2-3 cells, differently from control cells, showed a marked chromatin condensation and cell shrinkage identified by Hoechst 33342 staining, which are typical signs of apoptosis activation (Fig. 3*A*). Moreover, a 45% increase of the 17-kDa caspase 3 cleavage fragment was observed in K-Neu2-3 cells, with respect to mock and Neu2 (Ala → Asp) cells (Fig. 3, *B* and *C*), indicating an enhanced activation of caspase 3 and, therefore, of the apoptotic process.

Finally, serum starvation determined a marked redistribution of cytochrome *c* from the mitochondria to the cytosol, 67% greater in K-Neu2-3 cells than control cells (Fig. 3, *B–D*). These findings are consistent with the notion that the expression of Neu2 enhanced the sensitivity to apoptotic signals.

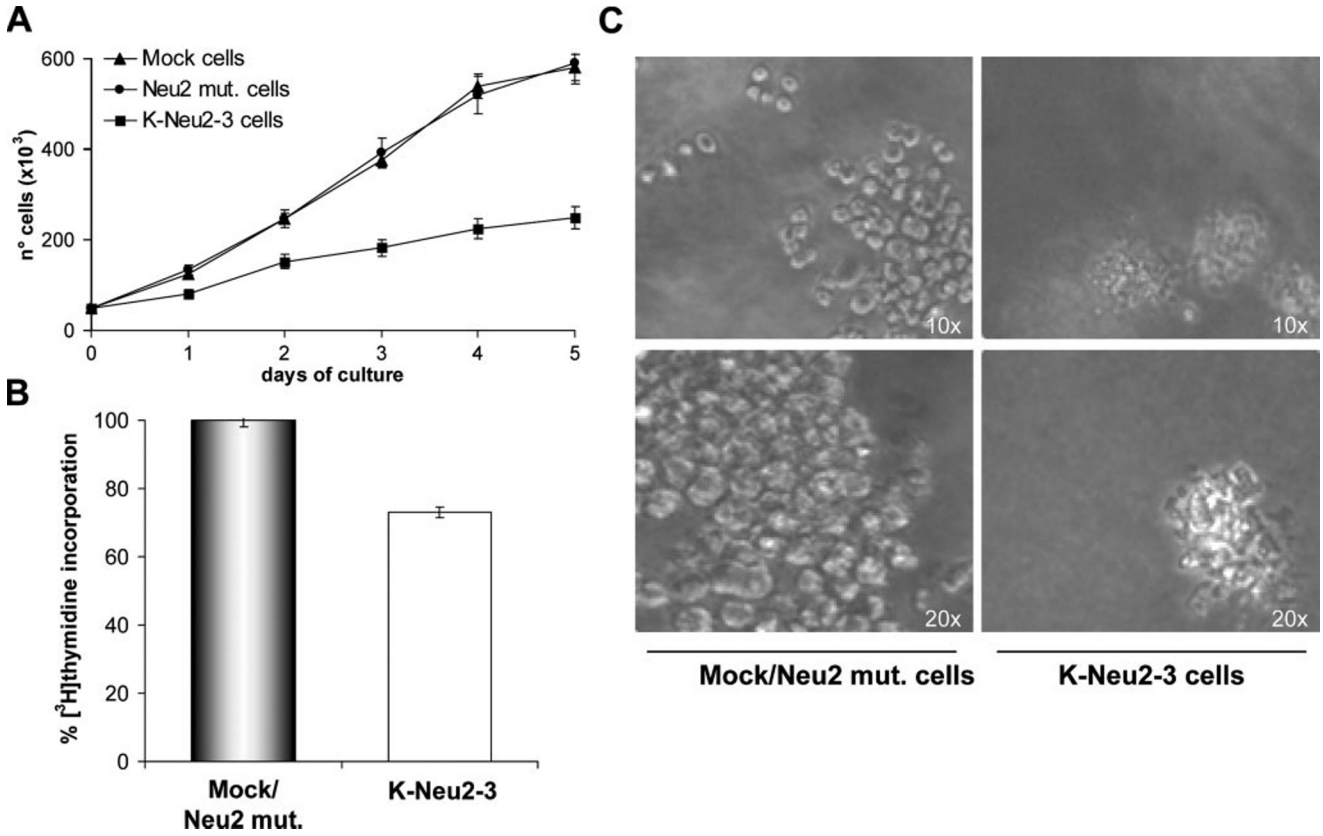

FIGURE 2. **Effects of Neu2 expression on the proliferation rate of K562 cells.** *A*, mock and Neu2 mutant transfected cells, as controls, and K-Neu2-3 cells proliferation curves are shown. Cell viability was determined by Trypan Blue dye exclusion assay. *B*, percentage of [^{3}H]thymidine incorporation in Mock and Neu2 mutant transfected cells, in comparison with K-Neu2-3 cells. The data are the means ± S.D. of six different experiments. Significance according to Student's test, $p < 0.001$. *C*, phase contrast microphotographs of colonies constituted by Mock, Neu2 mutant transfected and K-Neu2-3 cells in a semi-solid culture medium containing agar, after 2 weeks, at 37 °C. The images were obtained by a phase contrast microscope (IX50 Olympus). Original magnification, ×10 and -20.

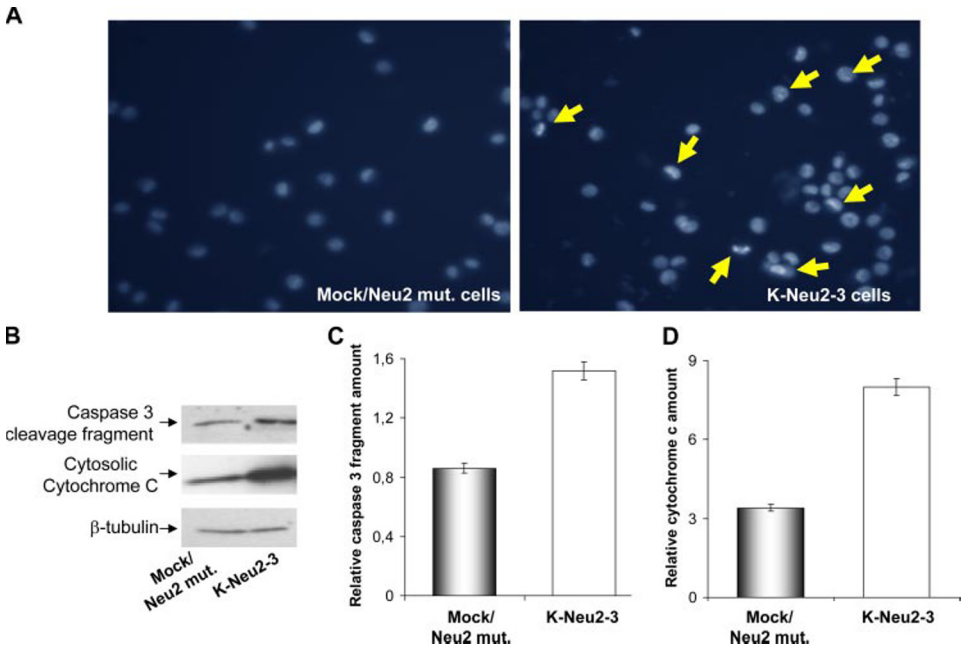

FIGURE 3. **Effects of Neu2 expression on the apoptosis resistance of K562 cells.** Mock, Neu2 mutant transfected, and K-Neu2-3 cells were cultured for 72 h, under serum depletion conditions, as apoptotic stimulus, and then analyzed for apoptosis extent. *A*, phase contrast microphotographs of control and K-Neu-3 cells after staining with Hoechst 33342. The images were taken with IX50 Olympus microscope. Original magnification, ×10. *B*, the levels of caspase 3 cleavage fragment and cytosolic released cytochrome *c* were assessed by Western blot. *C* and *D*, the quantitative data are reported in histograms. β-tubulin was used as internal control. Data are the means ± S.D. of six different experiments. Significance according to Student's test, $p < 0.001$.

Neu2 Induces a Down-regulation of the Anti-apoptotic Genes Bcl-2 and Bcl-XL—We then decided to investigate the possible cause of the observed susceptibility to apoptotic stimuli starting from mitochondrial apoptotic regulatory proteins. We examined the mRNA levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL and the pro-apoptotic protein Bad by real time PCR using β-actin as internal control for normalization. Our studies showed a marked down-expression (80%) of Bcl-2 mRNA in K-Neu2-3 cells (Fig. 4*A*), with respect to the control cells. Western blot analysis of K-Neu2-3 cells homogenates clearly showed a 95% decrease of Bcl-2 protein content compared with controls (Fig. 4*B*), indicating an almost complete disappearance of this anti-apoptotic factor because of the presence of Neu2 activity.

Moreover, we observed a 30% decrease in the level of Bcl-XL (Fig.

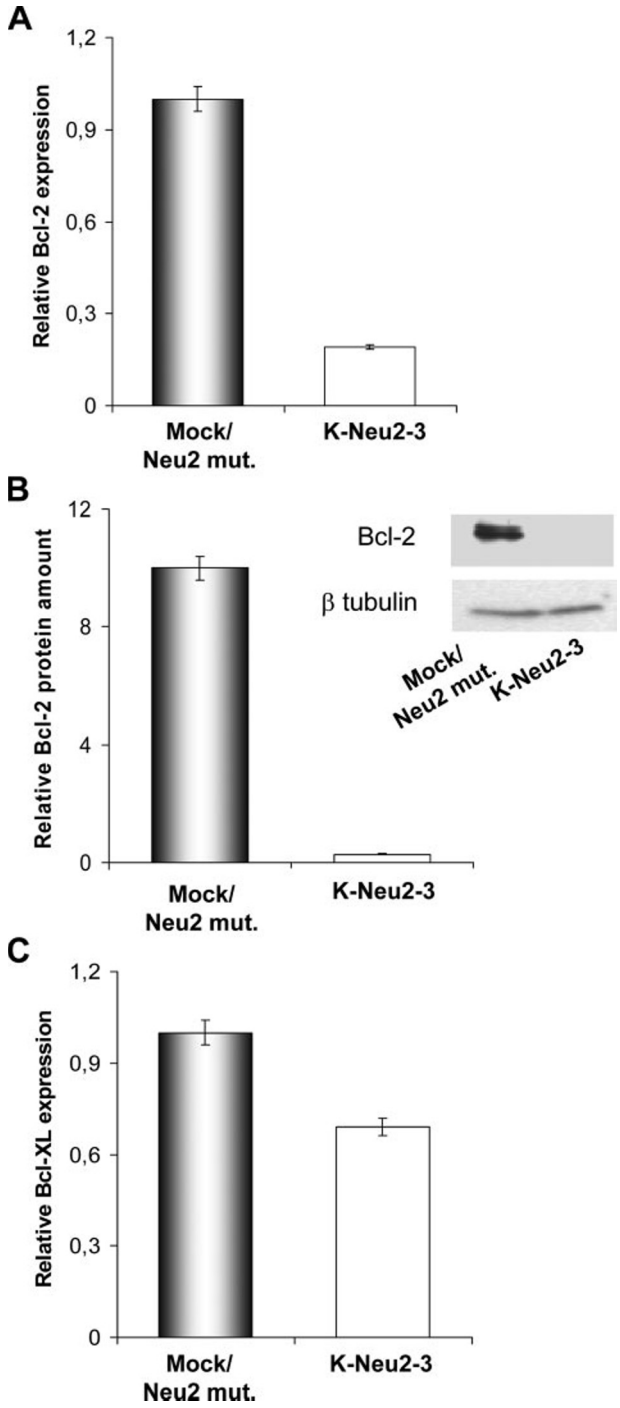

FIGURE 4. **Down-regulation of Bcl-2 and Bcl-XL expression level in K-Neu2-3 cells.** *A*, real time PCR analysis of Bcl-2 mRNA content in K-Neu2-3 cells in comparison with mock and Neu2 mutant transfected cells. *B*, Western blot determination of Bcl-2 protein amount in mock, Neu2 mutant transfected, and K-Neu2-3 cells. *C*, real time PCR analysis of Bcl-XL mRNA level in K-Neu2-3 cells, in comparison with mock and Neu2 mutant transfected cells. β-Actin and β-tubulin were employed as internal control for mRNA and protein analyses, respectively. The data are the means ± S.D. of five different experiments. Significance according to Student's test, $p < 0.001$.

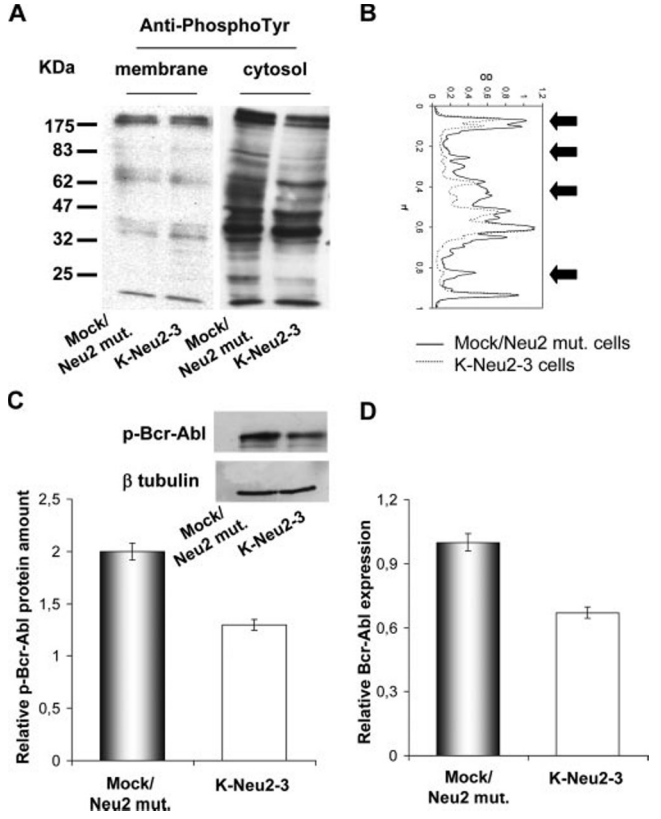

FIGURE 5. **Bcr-Abl autophosphorylation/activation and expression in K-Neu2-3 cells.** *A*, tyrosine phosphorylation pattern of membrane and cytosolic proteins extracted from mock, Neu2 mutant transfected, and K-Neu2-3 cells. The membranes blotted were probed with an antibody against phosphotyrosine. *B*, the membranes were subjected to densitometric analysis, employing Quantity One software (Bio-Rad). *C*, the autophosphorylation/activation extent of Bcr-Abl was performed by Western blot, employing an antibody against the phosphotyrosine 177 of Bcr, and the quantitative data, normalized to β-tubulin content, are represented in the histogram. *D*, real time PCR analysis of Bcr-Abl mRNA level in K-Neu2-3 cells in comparison with mock and Neu2 mutant transfected cells. β-Actin was used as internal control. The data are the means ± S.D. of four different experiments. Significance according to Student's test, $p < 0.001$.

4*C*) that plays a fundamental role in apoptosis protection in myeloid leukemic cells (11). Finally, we analyzed the expression of the pro-apoptotic protein Bad, but we did not observe any significant change between K-Neu2-3 cells and control cells (data not shown). Overall, the reduction of Bcl-2 and Bcl-XL proteins supports the notion of a direct involvement of Neu2 in the apoptotic/anti-apoptotic regulating pathways.

Neu2 Impairs the Content and the Activity of Bcr-Abl and Src Kinases, Decreasing the Tyrosine Phosphorylation Level of Intracellular (Cytosolic) Proteins—To investigate the molecular mechanism underlying the decrease of anti-apoptotic proteins in K-Neu2-3 cells, we evaluated the phosphorylation level of membrane and cytosolic proteins, as an indication of events pertaining the intracellular phases of signaling. As shown in Fig. 5*A*, cytosolic tyrosine phosphoprotein levels revealed with an anti-phosphotyrosine antibody by Western blot were markedly reduced in K-Neu2-3 cells. Particularly (Fig. 5*B*), a prominent band above 180 kDa, several proteins near 80 kDa, between 60 and 50 kDa, and near 30 kDa displayed decreased phosphotyrosine content. Conversely, the pattern of tyrosine phosphorylation in membrane-bound proteins did not provide significant changes depending on the presence of Neu2 activity (Fig. 5*A*). We therefore turned our attention to those kinases involved in determining apoptosis protection and uncontrolled proliferation (10), beginning with Bcr-Abl, which is known to play a key role in these processes.

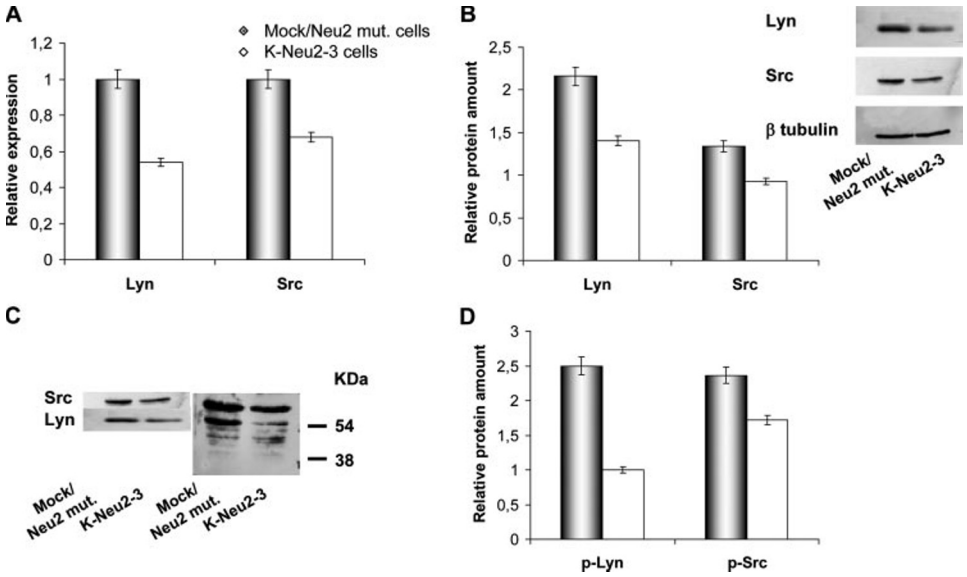

FIGURE 6. **Src and Lyn kinases expression and phosphorylation/activation in K-Neu2-3 cells.** Representative results for Src and Lyn mRNA (*A*) and protein (*B*) levels of mock, Neu2 mutant transfected, and K-Neu2-3 cells. *C* and *D*, the phosphorylation/activation extent of Lyn and Src was assessed using the phospho-Src family (Tyr^{416}) antibody, recognizing both phospho-Src (Tyr^{416}) and phospho-Lyn (Tyr^{396}). See "Results" for details. The densitometric quantification results are presented in the histogram. The data are the means ± S.D. of five different experiments. Significance according to Student's test, $p < 0.001$.

For this study, lysates from control and K-Neu2-3 cells were probed with a specific antibody for the tyrosine-phosphorylated form of Bcr-Abl. As depicted in Fig. 5*C*, the level of tyrosine phosphorylated Bcr-Abl underwent a 35% decrease in K-Neu2-3 cells, as compared with control cells. Concomitantly, the level of Bcr-Abl mRNA expression level showed also a 35% decrease in K-Neu2-3 cells (Fig. 5*D*). This indicates a connection between Neu2 activity and these intracellular signaling events.

Then we analyzed the content of some tyrosine kinases that act downstream of Bcr-Abl and are activated by this oncoprotein, particularly the Src kinases that play an essential role in Bcr-Abl-mediated malignant transformation and apoptosis resistance (40–43) and Lyn kinase, which is hugely expressed in leukemic cells (44). We observed that K-Neu2-3 cells showed a markedly decreased mRNA expression of both kinase genes (42% for Lyn and 32% for Src), compared with controls, as illustrated in Fig. 6*A*. The reduction of Lyn and Src genes transcription was accompanied by a decrease of the respective proteins, 35% for Lyn and 31% for Src, as shown in Fig. 6*B*. The level of tyrosine phosphorylation of Lyn and Src kinases at a site previously shown to be involved in their activation (45) was also explored. The antibody employed to reveal both phospho-Src (Tyr^{416}) and phospho-Lyn (Tyr^{396}) detected several activated members of the Src family. The identification of Lyn and Src was achieved reprobing the blots with Lyn or Src antibodies. A band detected by the phospho-Src antibody at 56 kDa corresponded to Lyn, whereas the other band revealed by the phospho-Src antibody at 60 kDa stained for Src (Fig. 6*C*). As shown in Fig. 6*D*, Src and, most importantly, Lyn phosphorylation/activation was reduced in K-Neu2-3 cells by 27 and 60%, respectively.

Neu2 Promotes Alterations on the Sialoglycoprotein Profile—To identify a possible molecular link between Neu2 action and alterations of tyrosine kinase-mediated signaling in K562 cells, we examined the modifications induced by Neu2 transfection to its putative endogenous substrates, namely sialosphingolipids (gangliosides) and sialoglycoproteins. Despite the fact that gangliosides are known to be mainly inserted in the plasma membrane, whereas Neu2 is localized in the cytosol, we checked the sphingolipid profile of K-Neu2-3 and control cells, through metabolic labeling with [^{3}H]sphingosine, which is their common precursor. We did not find any significant difference in the ganglioside pattern between K-Neu2-3 and control cells, except for a 30% increment of glucosylceramide (data not shown). The reason for this increase of glucosylceramide is under current exploration.

The ability of Neu2 to hydrolyze sialylated glycoproteins was assessed for both membrane and cytosolic proteins separated by SDS-PAGE and transferred onto PVDF membranes, with *S. nigra* agglutinin and *M. amurensis* agglutinin lectins, which are specific for α2–6 and α2–3 sialic acid linkage, respectively. Cell lysates treated with *S. nigra* agglutinin lectin did not show any significant difference between K-Neu2-3 and control cells, coherently with previous *in vitro* experimental data that exclude any Neu2 activity on glycoproteins carrying α2–6-linked sialic acid (36).

Conversely, the appearance (by transfection) of Neu2 in K562 cells caused a marked modification of the pattern of sialoglycoproteins containing α2–3-linked sialic acid residues. As illustrated in Fig. 7*A*, the major pattern changes concern cytosolic proteins. In K-Neu2-3 cells three proteins between 66 and 45 kDa underwent a marked loss of sialic acid from 50 to 70% compared with controls (Fig. 7*B*), indicating these sialoglycoproteins as the possible links between Neu2 activity and the susceptibility to apoptosis of K562 cells. Regarding membrane proteins, the desialylation process was evident only for a sialoglycoprotein of about 60 kDa of molecular mass, as shown in Fig. 7*A*.

DISCUSSION

Increasing understanding of the molecular bases of cancer has undoubtedly demonstrated that it is the inappropriate expression and activity of particular proteins involved in intercellular and intracellular signaling networks that causes radical changes in cell behavior, enabling prolonged cell survival and unlimited proliferative capacity. In the last years, a growing body of evidence supported the notion that glycoconjugates and glycosylation processes are involved in determining altered functionalities of signal proteins and, as consequence, aberrant cellular signaling (13, 16). Particular interest was devoted to

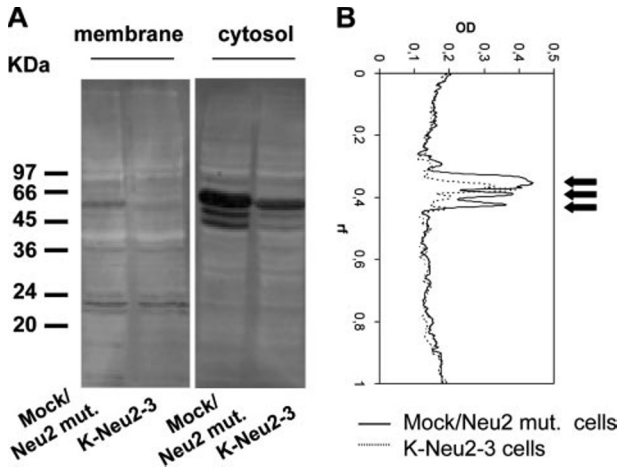

FIGURE 7. **Sialoglycoprotein profile of K-Neu2-3 cells.** Membrane and cytosolic proteins were separated by ultracentrifugation of lysates from mock, Neu2 mutant, and K-Neu2-3 transfected cells. After separation on 10% SDS-PAGE and transfer onto PVDF membrane, sialoglycoproteins were revealed employing the *M. amurensis* agglutinin lectin, specific for α2–3 sialic acid linkage. *A*, sialoglycoprotein profile assessed by Western blot. *B*, densitometric profile determined by Quantity One software (Bio-Rad).

sialoglyconjugates, sialoglycolipids, and sialoglycoproteins and the enzymes affecting sialylation, like sialidases (13–15, 19).

Chronic myeloid leukemia is a paradigm of how an uncontrolled and perpetually "switched on" tyrosine kinase, like the fusion-protein Bcr-Abl, could turn normal cells into malignant ones (10). Thus, considerable efforts have been made for understanding the molecular mechanisms underlying the transforming Bcr-Abl activity to possibly develop new therapeutic approaches, although several aspects are still obscure (6–12). However, the possibility of a direct involvement of glycoproteins, glycosphingolipids, and enzymes governing glycosylation processes in chronic myeloid leukemia was never explored.

These considerations constituted the preamble of our study, which was aimed at evaluating the effects of transfection of cytosolic sialidase Neu2 in the biochemical phenotype of a chronic myeloid leukemic cell line, K562 cells, which do not express this enzyme. Neu2 gene transcription seems to be an extremely regulated process, particularly linked to differentiation events (29, 46, 47), and several tumoral cells generally with a high degree of malignancy (27, 28), including leukemic cells (29), are lacking Neu2 sialidase.

Our results indicate that the expression of Neu2 by K562 cells has a considerable impact on the extent of the malignant features triggered by Bcr-Abl. First of all, the presence of Neu2 in K562 cells caused a marked reduction of the proliferation rate (−55%). Moreover, Neu2 transfected K562 cells showed a partially restored apoptosis susceptibility toward death signals such as serum deprivation.

The molecular bases of these behavioral modifications were firstly searched analyzing the processes that control the expression of mitochondrial anti-apoptotic regulatory proteins. We observed that, following Neu2 expression, both the most decisive anti-apoptotic factors, Bcl-XL and Bcl-2 (9), underwent a remarkable decrease of their mRNA levels (−30% and −80%, respectively), resulting in an almost total disappearance of the protein Bcl-2. To our knowledge, this is the first evidence for a precise involvement of Neu2 sialidase in the control of cell proliferation and apoptosis.

The modifications pertaining to the anti-apoptotic proteins Bcl-2 and Bcl-XL, which we revealed in Neu2 expressing K562 cells, constitute the prelude of significant alterations caused by the Neu2 presence in the complex system of tyrosine kinases governing cell proliferation and apoptosis. First of all, Neu2 expression led to a partial but significant inhibition of Bcr-Abl tyrosine kinase activity (−35%), as a consequence of the reduced autophosphorylation of the protein at Tyr^{177}, the site of the Grb2/Sos guanine nucleotide exchange complex binding (11). The reduction of the kinase activity of Bcr-Abl was associated to a parallel decrease (−35%) of its gene expression. This result is consistent with published data indicating that the inhibition of the tyrosine kinase activity of Bcr-Abl achieved by the therapeutic agent Imatinib also caused a reduction of its protein content (48). The loss of Bcr-Abl activity could be directly linked to the reduced expression of Bcl-XL, which is transcriptionally activated by Stat5, a factor up-regulated by Bcr-Abl (11).

The reduced activity of Bcr-Abl, in Neu2 expressing K562 cells, resulted to affect the expression/activation of the Src kinase family members that act downstream of Bcr-Abl (49). A particular relationship links the oncogenic activity of Bcr-Abl with the Src kinases, especially Lyn, which supports several anti-apoptotic and growth promoting properties of the oncoprotein (50). Recent data indicated that Lyn is clearly implicated in Bcl-2 up-regulation and, most importantly, is involved in a form of Imatinib resistance (41, 42).

In Neu2-expressing K562 cells, the signaling mediated by Src and Lyn was dramatically impaired as demonstrated by a reduced phosphorylation of Src and, most importantly, Lyn, at the amino acid site known to be strategic for their activation (45). Importantly, these effects were accompanied by a marked decrease of Src and Lyn mRNAs, by 32 and 42%, respectively, paralleled by the correspondent reduction of their protein levels, by 31 and 35%, respectively.

Summing up, these results provide clear evidence for the existence of a direct connection between sialidase Neu2 activity and Bcr-Abl activity with marked attenuation of the signaling pathways triggered by Bcr-Abl. Ultimately, the cytosolic tyrosine-phosphorylated proteins of the Src family, particularly Lyn, markedly weakened their activities, resulting in reduced expression of the Bcl-2 genes (9) and the facilitation of the apoptotic and anti-proliferative programs. All of these effects can be considered as directly associated to the catalytic activity of Neu2. In fact, the transfection with a mutant inactive form of Neu2 gave the same results as mock transfected K562 cells, that is K562 cells with unmodified malignant properties.

Of course, the link between Neu2 sialidase activity and the various steps of the signaling cascade leading to susceptibility of K562 cells to apoptosis is expected to be represented by a glycoconjugate that depending of its degree of sialylation acts as a switch off/on of a presumably upstream step of the above mentioned signaling cascade. The Neu2 transfected into K562 cells was not only able to efficiently recognize, *in vitro*, different sialylated compounds also at a neutral pH range, but also to markedly modify *in vivo* the sialylation level of three cytosolic pro-

teins with a molecular mass in the range 45–66 kDa. The sialic acid content of these proteins exhibited a marked decrease. Conversely, endogenous sialosphingolipids did not show any significant change, following Neu2 expression. This finding is not surprising because these glycoconjugates reside on the plasma membrane (external leaflet), whereas the enzyme is located in the cytosol.

Thus, we suggest that desialylation of key glycoproteins may be the cause of the effects triggered by Neu2 on Bcr-Abl and its tyrosine kinase activity. The separation and identification of these glycoproteins is under investigation. Numerous proteins have been shown to interact with Bcr-Abl (11); however, only few of them have been identified as modulators of the Abl tyrosine kinase. Only recently, a family of Abl interactor (Abi) proteins that interact with the SH3 domain of Abl and modulate the kinase activity has been identified (51, 52). The loss of expression of Abi proteins seems to be a component of Bcr-Abl-positive leukemia (53). Therefore, the possibility that an oversialylated glycoprotein inactive in the leukemic condition could be switched to a functional protein by Neu2 expression, thus promoting attenuation of malignancy, may also constitute an appealing working hypothesis for designing novel therapeutic approaches for chronic myeloid leukemia.

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