p53 loss of function enhances genomic instability and accelerates clonal evolution of murine myeloid progenitors expressing the p^{210}BCR-ABL tyrosine kinase

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Background and Objectives. The p210 bcr-abl fusion protein has a key role in the pathogenesis of chronic myeloid leukemia (CML). However, its influence on disease progression to blast crisis is marginal and mostly due to its effect of impairing the genomic stability of clonal myeloid progenitors through pathways still largely unknown.

Design and Methods. To elucidate the role of p53 in CML progression we generated, from the 32D murine myeloid cell line, several clones co-expressing the E6 product gene of human papilloma virus (HPV) 16, which abrogates p53 function, and a temperature-sensitive bcr-abl construct encoding a fully active p210 protein only at the permissive temperature of 33°C.

Results. Co-expression of the two proteins resulted in a significant enlargement of the G_2/M phase of cell cycle and in the appearance of a poly-aneuploid cell population. Furthermore, with continuous *in vitro* passages the p210 tyrosine kinase became dispensable for growth. Increased levels of cyclin B_1 and enhanced activity of its associated cyclin-dependent kinase (cdc2) became apparent during the clonal evolution of p210 bcr-abl-transduced 32D cell clones lacking p53.

Interpretation and Conclusions. The acceleration of clonal evolution of p210 bcr-abl-transduced 32D myeloid progenitors associated with p53 functional abrogation is consistent with oncosuppressor loss having a key role in CML progression. This would allow emergence of additional genomic aberrations which would lead to the fully transformed phenotype of blast crisis. Deregulated activity of the cyclin B1-cdc2 complex may be involved in the loss of temporal co-ordination of mitotic events and further free the barrier to genomic instability of CML clonal myeloid progenitors lacking p53.

Key words: p53, chronic myeloid leukemia, HPV 16 E6, cell cycle checkpoints, cyclin B.

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Chronic myeloid leukemia (CML) is a myeloproliferative disorder arising from the multipotential hematopoietic stem cell and leading to the illegitimate expansion of a clonal myelopoiesis still capable of completing maturation processes.[1] Within the Philadelphia (Ph1) chromosome, the cytogenetic hallmark of CML, most of the *c-abl* proto-oncogene on chromosome 9 is fused to the amino-terminal of *bcr* on chromosome 22.[2] The tyrosine kinase of the p210 bcr-abl protein, constitutively activated by the oligomerization of a coiled-coil domain at the N-terminus of bcr, is the causative event of CML.[3,4] The initial indolent phase of CML, the chronic phase, inevitably progresses to the acute terminal phase, the blast crisis, marked by the emergence and selection within the Ph1$^+$ hematopoiesis of more aggressive clone(s) arrested at an early stage of either myeloid or lymphoid differentiation. The appearance of additional, non-random chromosomal aberrations in blast crisis directs the clonal evolution of bcr-abl-rearranged myelopoiesis towards superimposed genetic events.[5,6] Indeed, previous studies are consistent with an intrinsic genomic instability of CML myeloid progenitors, possibly associated with DNA repair defects, such as deletion of p16, inactivation of the retinoblastoma gene product, enhanced expression of polymerase β (the least faithful mammalian DNA polymerase), inhibition of the catalytic function of xeroderma pigmentosum group B protein or downregulation of the DNA-dependent protein kinase complex). However, p53 is the gene most often involved in CML progression. Its oncosuppressor function may be lost in consequence of point mutations at critical residues, deletions or rearrangements. Moreover, the frequent and preferential loss of residual p53 allele in bcr-abl-transduced myeloid progenitors suggests that it can be unstable under conditions that do not preclude its function.[7-13]

p53 plays a key role in cellular responses to genotoxic stress. It contributes to the maintenance of genomic integrity by transactivating downstream target genes involved in cell cycle arrest and in nuclear excision repair of genomic DNA or in the induction of apoptotic cell death.[14]

We investigated the combined effects of p210 bcr-abl tyrosine kinase and p53 loss of function induced by the human papilloma virus (HPV)-16 E6 protein gene (which functionally inactivates p53 by binding and targeting its product at the proteasome for ubiquitin-dependent degradation) on cell cycle checkpoints and

cell ploidy.[15] Co-expression of the two genes, besides abrogating the G_1/S checkpoint as the single genes did, reinforced the G_2/M checkpoint, progressively increased G_2/M size and allowed the appearance of a poly/aneuploid cell population. The overexpression of cyclin B1 and the enhanced activity of cdc2 kinase, both under the control of p53, were associated with long-lasting expression of p210 bcr-abl tyrosine kinase and HPV E6. They probably play a role in the emergence from mitosis of cells bearing gross karyotypic aberrations.

Design and Methods

Cell lines and culture conditions

The 32 cell line is a murine myeloid progenitor cell line strictly dependent on interleukin (IL)-3 for growth and is generated from the non-adherent fraction of long term bone marrow cultures.[16] The 32D PuroERE6 cell line expressing the E6 protein gene of HPV 16 was kindly provided by E.V. Prochownick (University of Pittsburgh, PA, USA). Its characteristics have been published elsewhere.[17] We used electroporation at 0.25V/960mF (Equibrio Easyject, Optima) to express, in the 32D and 32D PuroERE6 cell lines, a temperature-sensitive (ts) mutant of bcr-abl construct subcloned in a pDG retroviral vector under the control of LTR promoter of the myeloproliferative sarcoma virus along with the neomycin phosphotransferase resistance gene (which allows selection in G418-additioned media) kindly donated by N. Carlesso (Massachusetts General Hospital, MA, USA) and described in more detail elsewhere.[18] Briefly, the ts-bcr-abl construct has a mutated abl domain; in this way it retains tyrosine kinase activity of its p210 protein at the permissive temperature of 33°C, but not at the non-permissive temperature of 39°C. Following electroporation, cells were allowed to recover in the presence of 10% WEHI 3 conditioned medium as the source of IL-3 for 48 hours and thereafter selected at 33°C in G418 (500 μg/mL)-additioned RPMI (Gibco) in the absence of IL-3. Individual cell clones were generated from ts-p210 bcr-abl-transduced 32D and 32D PuroERE6 by expanding single colonies grown at 33°C in methylcellulose in the absence of IL-3 and in the presence of G418. Cloning and expansion processes took about 50 days. Polyclonal cell populations expressing either empty vectors (pDG and PuroER) were generated by the same method. They were kept in RPMI to which WEHI 3 conditioned medium had been added.

Gamma irradiation was delivered by a ^{60}CO source with filtration adjusted to deliver a dose of 0.052 Gy/min at the flask surface. Cell cycle distribution was assayed 16 hours after exposure to a single radiation dose of 4 Gy.

RNA analysis

Total RNA was isolated by the RNeasy kit from Quiagen, according to the manufacturer's instructions, quantified by optical density at 260 nm and assayed for integrity and DNA contamination by electrophoresis in 2% agar under denaturing conditions. Details of reverse transcription (RT) and the polymerase chain reaction (PCR) were given in a previous paper.[19] According to the principles of competitive PCR, the ratio between the amplification products was linearly correlated to the input amount of competitor. The transcript molecule number/μg total RNA were, thus, calculated according to the equation of the line fitting the experimental points. Each quantification was repeated three times in at least three separate experiments for each cell clone.

Protein analysis and immunocomplex kinase assays

Immunocytochemical analysis of p210 bcr-abl expression was performed on cytospin slides following overnight incubation with an anti-abl monoclonal antibody (Santa Cruz) and 2 hour incubation with a secondary antibody (Daco). Alkaline phosphatase anti-alkaline phosphatase (APAAP) from Daco was used as the revealing system. Five hundred to 700 cells were scored to assess the percentage of positive cells. Immunoprecipitation and immunoblotting were performed according to standard techniques.[18] Equal protein amounts were either resolved in SDS-PAGE or immunoprecipitated with primary antibodies (anti-cdc2 from Upstate Biotechnology and anti-cyclin B_1 from Santa Cruz) in the presence of CNBr-activated sepharose 4B (Pharmacia) and then resolved in SDS-PAGE. Blots were then transferred in nitrocellulose (Schleicher and Schuell), labeled with anti-p53, anti-G3PDH housekeeping gene, anti-cyclin B_1 (Santa Cruz) and anti-cdc2 antibodies, with the proper secondary antibodies and visualized by an enhanced chemoluminescence (ECL) detection system (Amersham Corp). Immunocomplex kinase assays were performed on anti-cdc2 immunoprecipitates incubated for 30' at 37°C in the presence of histone H1 (Upstate Biotechnology) as substrate and 10 μCi/μL [γ-^{33}P] ATP (Amersham), eluted through phosphocellulose filters (Pierce), washed three times in 500 mM phosphoric acid to eliminate the unbound radionuclide, resolved in SDS-PAGE and detected by autoradiography using Pierce films.[20]

Cytofluorimetric analysis of cell cycle distribution

Cell cycle distribution was evaluated by bromodeoxyuridine (BrdU) staining and propidium iodine (PI) uptake in cells fixed in 70% ethanol, incubated in denaturing acid solution (4N HCl,

0.5% Triton-X 100) with the primary anti-BrdU and secondary FITC-conjugated antibodies (Sigma) for 30' or with the secondary anti-BrdU antibody alone, (to have a measure of aspecific cell fluorescence and with PI (5 μg/mL) for 45'. BrdU staining and PI uptake were assayed by cytofluorimetric analysis and dedicated software (Cell Quest from Becton-Dickinson).

Results

Co-expression of HPV 16 E6 product gene and bcr-abl under culture conditions permissive for p210 tyrosine kinase abrogate the G1/S checkpoint and promote the emergence of a poly/aneuploid cell population

The ts-p210 protein expressed in 32D and 32D PuroERE6 cell lines has constitutive tyrosine kinase activity at the permissive temperature of 33°C, but lacks it at the non-permissive temperature of 39°C.[18] Accordingly, 32D ts-p210 and 32D PuroERE6- ts-p210 cell lines became IL-3 independent when kept at 33°C, but IL-3 was mandatory to allow cell survival at 39°C. Five and 7 independent cell clones were generated from the polyclonal 32D ts-p210 and 32D PuroERE6-ts-p210 cell lines by expanding, at 33°C, single colonies grown in methylcellulose deprived of IL-3 at 33°C. No significant differences in E6 gene transcription were seen in 32D PuroERE6 and 32D PuroERE6-ts-p210 cell clones (*data not shown*). To investigate early events that have might occurred during the 50-day interval required for the expansion of individual cell clones expressing p210 bcr-abl alone or together with the E6 protein, we used polyclonal 32D ts-p210 and 32D PuroERE6- ts-p210 cell lines kept in the absence of IL-3 at 33°C or switched to 39°C in medium to which IL-3 had been added 24 hours before. G418-resistant/factor-independent polyclonal cells expressing either the E6 and ts-p210 bcr-abl genes together or the latter gene alone started emerging 15 and 22 days, respectively, after electroporation. By that time, more that 80% cells were positive for p210 bcr-abl immunostaining and this percentage was not significantly different after culture switching to 39°C in medium to which IL-3 had been added (Figure 1A and data not shown). p210 bcr-abl expression levels were measured by means of a competitive PCR strategy. The competitive PCR technique is based on co-amplification of the sample template (target) together with increasing amounts of a DNA fragment (competitor) sharing with the target the primer recognition sites, but differing in size.[19] No significant differences in p210 bcr-abl expression level relative to the E6 product gene expression were apparent ($p<0.1$). The number of p210 bcr-abl transcripts ranged between 4 and 5×10^8 molecules/μg total RNA by the time a p210 bcr-abl-positive/G418 resistant/IL3-independent cell population became prominent and continued to rise thereafter (up to $\sim6\times10^8$ in polyclonal cell populations assayed at the end of the 6th week after electroporation and up to $3.5\text{-}6\times10^8$ in individual cell clones assayed at the end of the 8th week) (Figure 1B).

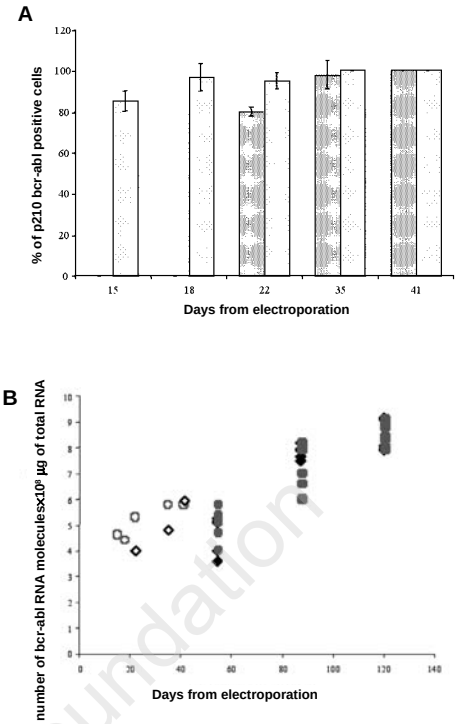

Figure 1. A. Time course analysis of p210 bcr-abl-positivity in polyclonal 32D and 32 PuroERE6 cell lines after electroporation of the ts-p210 bcr-abl construct. The p210 bcr-abl immunostaining was performed by the APAAP technique. Results are the means ± SD of three repeated experiments. 32Dtsp210:□; 32DPuroERE6-tsp210:■. B: Time-course analysis of p210 bcr-abl expression levels. The ◇ symbol marks bcr-abl transcript levels of 32Dtsp210 polyclonal cell line (open symbols) and individual cell clones (closed symbols). The □ symbol marks bcr-abl transcript levels of 32DPuroERE6-tsp210 polyclonal cell line (open symbols) and individual cell clones (closed symbols). P210 bcr-abl levels were quantified by competitive PCR. All values represent the means of three repeated experiments. SDs did not exceed 10% (*data not shown*).

p53 function was assayed by the induction of its product by low dose (4 Gy)/low dose-rate (0.052 Gy/min) γ-irradiation. Since p53 induction by ionizing radiation is strictly dependent upon the wild-type conformation of the gene, we preliminarily excluded the presence of p53 point mutations in all cell types by directly sequencing the p53 coding

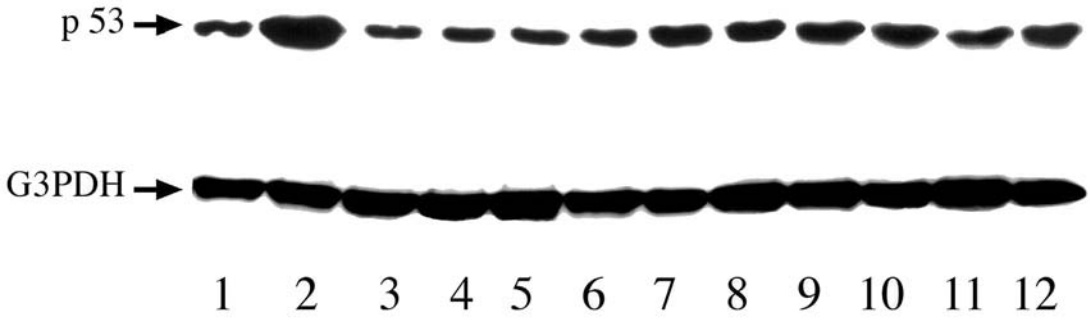

Figure 2. p53 induction by IRs. Western blotting was performed 16 hours after a single low dose (4 Gy) of low dose rate (0.052 Gy/min) γ-irradiation. Proteins from control and irradiated 32D, 32D PuroERE6, 32DPuroERE6-ts-p210 kept at 33°C, 32DPuroERE6-ts-p210 kept at 39°C, 32Dts-p210 kept at 33°C and 32Dts-p210 kept at 39°C were sequentially loaded. G3PDH housekeeping was used as an internal control for protein loading.

sequence from the 5th through to the 9th exon, the region accounting for most mutations in cancer cells and cancer cell lines (*data not shown*).[21] p53 was induced to equivalent levels in control (parental and empty vector-transduced) cell lines but not in 32D PuroERE6 and 32D PuroERE6-ts-p210 cells kept at either temperatures (33°C or 39°C). Interestingly, the p53 radio-induction was abrogated in 32D ts-p210 at 33°C, but not at 39°C (Figure 2).

p53 radio-induction was associated with G_1 arrest in the parental 32D cell line while, as expected, it was not in the 32D PuroERE6 cells. In this latter cell type the bypassing of the radio-induced G_1 arrest was associated with a reinforcement of G_2 arrest (Figure 3, top panels). p210 tyrosine kinase had effects similar to E6 on the sizes of both G_1 and G_2. After exposure to ionizing radiation, 32D ts-p210 cells kept at 33°C proceeded throughout the G1/S boundary and became arrested in G_2/M. They were, conversely, arrested in the G_1 phase when kept at 39°C (Figure 3, middle panels). Notably, radiation-induced G_2 arrest was significantly (p<0.01) enlarged by co-expression of the two genes in cells kept at 33°C, but not in cells kept at 39°C, in which G_2 arrest was the same size as that induced by the E6 gene alone (Figure 3, bottom panels).

The cell cycle distribution of polyclonal 32D ts-p210 and 32DPuroERE6-ts-p210 was investigated for 6 weeks after electroporation. Parental and empty vector-transduced 32D, and 32D PuroERE6 cell lines conserved the cell cycle distribution shown in Figure 3 over this time. A slightly significant (p<0.05) reduction of G_1 size in the 32D ts-p210 cell line kept at 33°C was consistent with previous reports (Figure 4A, lower panels).[22,23] In addition, the size of the G_2/M phase in the 32DPuro-

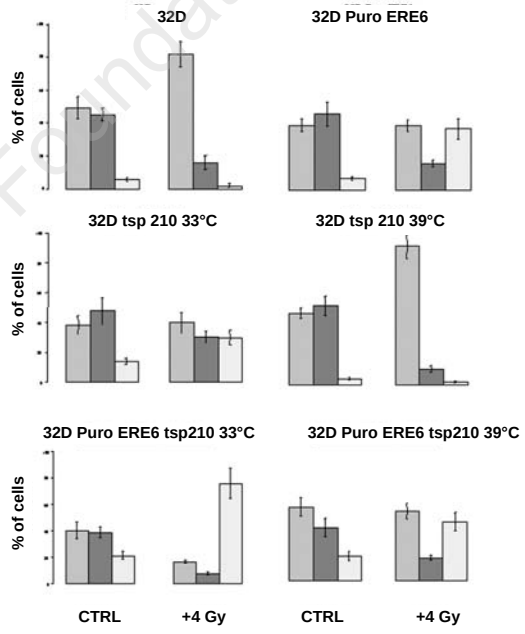

Figure 3. Radiation-induced changes in cell cycle distribution. Polyclonal 32Dts-p210 and 32DPuroERE6-ts-p210 were assayed for their cell cycle distribution 22 days after electroporation. See legend to Figure 2 for γ-irradiation dose and dose rate. G_0/G_1: ; S: ■; G_2/M: □.

ERE6-ts-p210 polyclonal cell population kept at 33°C progressively and statistically significantly (p<0.01) increased from the 35th day after electroporation. At that time, a poly/aneuploid cell population started emerging (Figure 4A, upper right

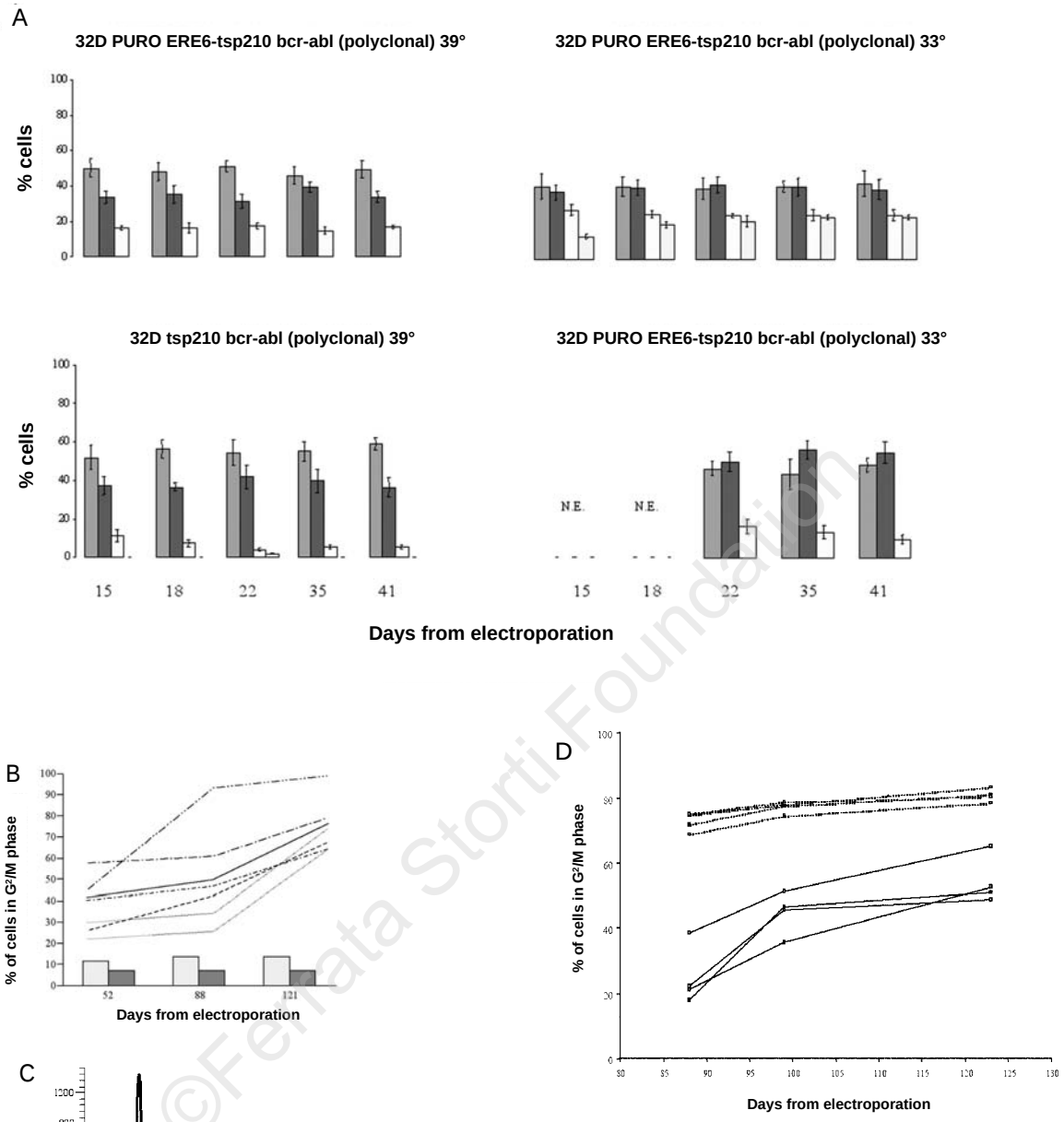

Figure 4. A.Time-course analysis of cell cycle distribution of polyclonal 32DPuroERE6-ts-p210 and 32Dts-p210 kept at 39°C (left panels) or at 33°C (right panels). See legend to Figure 3 for symbol specifications. Poly/aneuploid cells: . B. Time-course analysis of G_2/M size in 7 independent 32DPuroERE6-ts-p210 cell clones. The G_2/M size of 32Dts-p210 clone 7B (representative of 4 other of the same age) (□) and of polyclonal 32DPuroERE6 (■) are indicated in bars. C. Profiles of cell cycle distribution in polyclonal 32DPuroERE6 (upper panel), 22-day old polyclonal 32DPuroERE6-ts-p210 kept at 33°C (middle panel) and 52-day old polyclonal 32DPuroERE6-ts-p210 kept at 33°C (lower panel). Cell cycle phases were distinguished by cytofluorimetric analysis BrdU staining and PI uptake. D. Time-course analysis of the G_2/M size in subclones generated from individual 32DPuroERE6-ts-p210 clones 1B (continuous lines) and 4B (dotted lines). Subclones were generated by limiting dilution at 52nd day after electroporation.

panel). Both for G_2/M enlargement and the poly/aneuploidy of 32DPuroERE6-ts-p210 cell line were revoked by switching them to 39°C 24 hours before cell cycle analysis (Figure 4A, upper left panel, and *data not shown*). These results are consistent with the mandatory requirement of p210 tyrosine kinase for deregulated cell cycle progression and onset of mitosis in cells lacking p53.

The cell cycle distribution and ploidy of 7 independent 32DPuroERE6-ts-p210 cell clones were followed from the 7th up to the 18th week after electroporation. In spite of discrete differences consistent with their clonal origin, all of them exhibited a significant (p<0.01 or less) and progressively increasing enlargement of the G_2/M phase compared to 5 individual 32D ts p210 cell clones of equal age and to the 32D PuroERE6 cell line. The G_2/M size ranged between 24%-50% at the beginning of the 7th week and between 50%-80% at the end of the 18th week (Figure 4B). Moreover, cells with a polyploid or aneuploid DNA content started emerging and their percentage, although variable in individual cell clones, progressively increased (Figure 4C). Thus far, in all 7 32DPuroERE6-ts-p210 cell clones the G_2/M size and the number of poly/aneuploid cells remained steady after switching temperature from 33°C to 39°C (*data not shown*), suggesting that their clonal evolution became independent of p210 tyrosine kinase activity.

Finally, for 5 additional weeks we followed the cell cycle distribution of 4 independent subclones generated at limiting dilution from two 32DPuroERE6-ts-p210 cell clones exhibiting discrete differences in G_2/M size, namely 1B and 4B. Differences relative to G_2/M size and poly/aneuploidy of the two groups of subclones and cell clones from which they were originated were not significant (p<0.1) (Figure 4D). These findings support the concept that the genomic fragility which impedes most cells from attempting mitosis and lowers the surveillance over reduplicated DNA (allowing, in turn, some of them to re-enter the cell cycle without having properly completed mitosis) is an epiphenomenon of p210 tyrosine kinase activity associated with p53 loss.

The transcription rate of the bcr-abl fusion gene might be involved in faster clonal evolution of bcr-abl-transduced cells lacking p53. Clinical outcomes and experimental observations have, in fact, correlated the disease acceleration to chimeric gene amplification.[24] However, the p210 bcr-abl transcript levels did not exhibit significant (p<0.1) differences relative to E6 gene expression. Indeed, the p210 bcr-abl expression progressively increased with continuous *in vitro* passages and approximatley doubled within the 8th-17th week interval after electroporation, irrespectively of the co-expression of the two genes (Figure 1B).

Increased expression of cyclin B1 and enhanced activity of associated cdc2 kinase characterize the clonal evolution of cells expressing the p210 tyrosine kinase and lacking p53

Regulated progression throughout the cell cycle is driven by the sequential activation of a family of protein kinases composed of a catalytic and a regulatory domain: the cyclin-dependent kinases (cdks) and the cyclins, respectively. In mammalian cells progression through the G_2 phase and the onset of mitosis are under the control of two cyclin/cdk complexes, cyclin A/cdk2 and cyclin B/cdc2.

We sought to determine whether co-expression of HPV 16 E6 and p210 bcr-abl affected cyclin B_1 expression and cdc2 kinase activity.

To this purpose, we measured cyclin B_1 levels by immunoprecipitation/immunoblotting and cdc2 kinase activity by immunocomplex kinase assay on histone H1 as the substrate in the 32D parental cell line, in the 32DPuroERE6 polyclonal cell population, in the 32D ts-p210 clone 7B and in the 32DPuroERE6-ts-p210 cell clones 1B and 4B on the 88th day after electroporation. By that time, the G_2/M of the last two cell clones was already significantly enlarged and more than 20% and 30% of cells, respectively, were poly- and aneuploid (Figure 4B). Co-expression of the two genes was associated with a quite remarkable increase of cyclin B1 levels. Cdc2 activity was also higher in 32DPuroERE6-ts-p210 cell clones 1B and 4B than in the 32D parental cell line and the 32D cells expressing the E6 and the p210 bcr-abl genes alone. Conversely, cdc2 expression was not affected by the co-expression of the two genes (Figure 5).

Discussion

Genomic instability plays a key role in the clonal evolution of CML myelopoiesis towards its fatal outcome, the blast crisis. The p210 bcr-abl tyrosine kinase delivers a signal for unrestrained progression through the cell cycle and abrogates the arrest at discrete transition points known as cell cycle checkpoints. Thus, it is itself a cause of genomic instability of CML progenitors.[25] The loss of the p53 oncosuppressor might accelerate disease progression by impairing cell cycle checkpoints to a greater extent and further enhancing the genomic instability of clonal CML progenitors.[26]

Our study demonstrated that loss of p53 function induced by either E6 protein of HPV16 or by the p210 bcr-abl tyrosine kinase abrogated the G_1/S checkpoint and reinforced the G_2/M checkpoint. Our data are consistent with those of previous studies and account for the genomic instability associated with the expression of both proteins.[27-29] However, E6 and p210 expressed alone allowed 32DG cells to

maintain a stable, diploid genotype over intervals even longer than these shown in Figure 4B, while co-expression of the two genes led to the spontaneous accumulation of a prominent tetraploid cell population and to poly/aneuploidy. Both genes must contribute to the accelerated clonal evolution of cells co-expressing them. They abrogate the G_1/S checkpoint, the most powerful tool for DNA damage recognition and repair, and prolong the G_1/M arrest, the last chance to prevent reduplication of damaged DNA sequences. Moreover, they inhibit apoptotic cell death, which precludes the propagation of unrepairable genomic lesions.[30-32] In addition, in the absence of functional p53, the constitutive transduction of mitogenic signal(s) by the p210 tyrosine kinase might promote adaptation and let G_2/M-arrested cells to progress to further cell cycle phases even in absence of proper termination of previous mitoses.[33]

The bcr-abl amplification, which is the biomolecular event most often associated with CML progression, does not play any role in clonal evolution of 32DPuroERE6- ts-p210 cells.[24] The bcr-abl levels observed in 32D cell clones expressing the ts-p210 protein either alone or together with E6 did not, in fact, exhibit significant differences (Figure 1B). The matter of higher integration of the bcr-abl construct in 32D cell clones has been discussed in a previous work.[19] It is likely contingent upon the used technique, i.e. electroporation, and would account for the discrepancies between the fusion gene transcription rates in our p210 bcr-abl-transduced 32D cell clones and those seen by other groups and by ourselves in the CML hematopoietic cell lines, K562 and LAMA84 ([21] *and data not shown*).

The duration of the co-expression of the two genes influenced the requirement of p210 tyrosine kinase for the altered cell cycle distribution and ploidy of p210 bcr-abl-transduced 32D cell clones lacking p53. In fact, beyond the 7th week after electroporation both the G_2/M enlargement and the appearance of a poly/aneploid cell population were not revoked by switching the culture conditions from a permissive (33°C) to a non-permissive (39°C) temperature (Figures 4A and B). Moreover, the increase of a tetraploid cell population followed a fixed pattern (Figure 4D), supporting the behalf that the effects of long-lasting p210 tyrosine kinase expression in the absence of functional p53 on hematopoietic progenitor karyotypic integrity are not stochastic. Our results suggest distinct roles of the p210 bcr-abl in the transformation and progression of clonal CML hematopoiesis: the fusion protein might allow cells to take the first steps of leukemic transformation by abrogating multiple pathways devoted to the control of proliferation and apoptosis, a threatening condition for the maintanence of DNA integrity. The loss of function

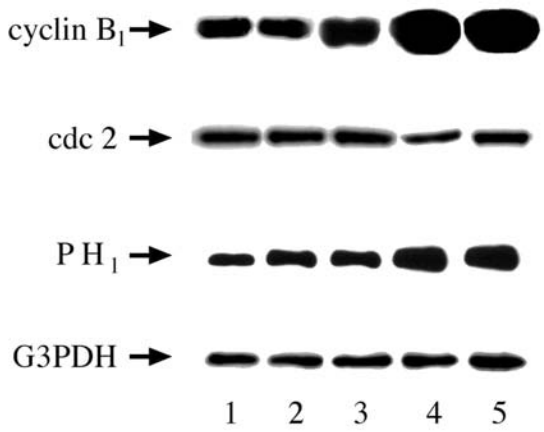

Figure 5. Cyclin B1 and cdc2 expression levels and cdc2 kinase activities on histone H1 substrate in parental 32D, polyclonal 32DPuroERE6, 32Dts-p210 clone 7B and 32D PuroERE6-ts-p210 clones 1B and 4B kept at 33°C (lanes 1, 2, 3, 4 and 5, respectively). The last three cell clones were assayed for protein levels and activities 88 days after electroporation. G3PDH was used as an internal control for immunoprecipitation.

of p53 would accelerate the outcome of additional, p210 bcr-abl-independent genomic aberrations which drive further transforming steps and lead to a malignant phenotype. Under this perspective, loss of p53 function might be a marker of a worse prognosis for CML patients and might even be associated with their unresponsiveness to the tyrosine kinase inhibitor, STI571.

Cyclin B1 overexpression and enhanced cdc2 activity were associated with the evolution of 32DPuroERE6-tsp210 cell clones (Figure 5). They might, at least partly, result from the expression of HPV E6 protein and consequent loss of p53 function, since cyclin B transcription and stability and cdc2 phosphorylation are both under p53 control, although the impact of latter event has so far been proven only in human fibroblasts.[34-37] Since HPV E6 itself affected neither cyclin B1 expression nor cdc2 activity, further studies are required to link p53 loss and p210 tyrosine kinase to these two regulatory signals of cell cycle progression in CML evolution. It is worth noting that deregulated expression of cyclin B, perhaps associated with early activation of its cdc2-associated kinase, provides a condition itself prone to mitotic catastrophes and is involved in the cell's predisposition to a tetra/aneuploid genotype.[38]

Intermediate genes might be involved in the synergic adverse effects of p210 tyrosine kinase and loss of p53 function on karyotypic integrity of hematopoietic progenitors. C-myc is the most likely candidate: this is, in fact, upmodulated by bcr-

abl in primary CML myeloid progenitors and cell lines and was required, in some instances, for bcr-abl-induced cell transformation.[39-41] Interestingly, the co-expression of c-myc and HPV16 E6 in the 32D cell line allowed the emergence from mitosis of cells with increasingly severe karyotype damage and poly/aneuploidy quite similar to these induced by E6 and p210 bcr-abl co-expression.[16] Furthermore, a recent work provided evidence for an inverse regulation of cyclin B1 by c-myc and p53.[41] In conclusion, cyclin B/cdc2 may be viewed as a critical point of convergence of p210 brc-abl and p53 loss in the accelerated progression of CML, and could be helpful in a prognostic classification of the disease.

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Pre-publication Report & Outcomes of Peer Review

Contributions

All co-authors gave substantial contributions to the conception and interpretation of data presented here, and to drafting and revising the article; all of them approved the article in its final form. In particular: GB, MB, LM, MM and LP set up and performed most experimental procedures; GM supervised the RNA analysis procedures; EB set the conditions for *in vitro* γ irradiation and provided the instrument calibration and dose/dose rate assessment; JSG and MB's contributions to the interpretation and critical appraisal of data were fundamental. The corresponding author MAS contributed to and supervised the laboratory work and drafted the proper instrument in its many versions, including the final one submitted to Haematologica for publication.

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Disclosures

Conflict of interest: none.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Francesco Lo Coco, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Lo Coco and the Editors. Manuscript received February 25, 2003; accepted May 7, 2003.

In the following paragraphs, Professor Lo Coco summarizes the peer-review process and its outcomes.

What is already known on this topic

Molecular pathways of clonal progression in Ph+ chronic myeloid leukemia are still under investigation.

What this study adds

The study proposes an experimental model in which the BCR/ABL oncoprotein and abrogation of P53 function co-operate in inducing genomic instability through interference with cell cycle checkpoints leading to additional chromosomal lesions.

Caveats

Whether the model is clinically relevant in the transition from chronic towards blastic phase in CML patients require further studies.