Reduction of Cell Proliferation Induced by PD166866: an Inhibitor of the Basic Fibroblast Growth Factor

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Cell proliferation control plays a key role in tumor development. The basic Fibroblast Growth Factor (bFGF), as well as other growth factors, is involved in several pathologies characterized by dysregulation of cell proliferation. In the present work the effects of PD166866, a very potent and selective tyrosine kinase inhibitor were evaluated. Cultured murine fibroblasts (the cell line 3T6) were used to assess the FGFR-1 inhibition mediated by PD166866. Evaluation of cell viability and molecular biology techniques were adopted. PD166866 controls negatively the bFGF/FGFR-1 system thus promoting a significant reduction of cell proliferation and loss of viability in 3T6 cells. The drug possibly controls proliferation via induction of apoptosis as evidenced by a relevant chromatin degradation. Conclusion: This study demonstrated that PD166866 might be used in the control of fibrotic proliferative diseases, as well as in other tumor pathologies.

Key Words: Fibroblast growth factor, Reduction of cell proliferation, Cell death

Fibroblast growth factors (FGFs) are part of a large group of proteins involved in different cell population growth control as well as in the normal cell differentiation processes. They have been found in both invertebrate and vertebrate metazoans. A high number of genes coding for FGFs were identified along with higly protein primary structure sharing more than 90% sequence homology at aminoacid level. In vertebrates the molecular weight of FGFs ranges from 17 to 34 kDa; most of them present a core constituted by six identical aminoacid residues with twenty-eight highly conserved additional residues. Ten residues in core interact with the FGF receptor (FGFr). Protein structure studies conducted on the acidic factor aFGF1 and bFGF2 have given an insight on their function/structure relationships (1-3).

Over-expression of growth factor as well as their receptors is involved in cell proliferation disorders, cancer progression being one of them (4-6). Fibroblasts and growth controlling factors are therefore the object of intensive oncological studies. In many epithelial tumors, as a matter of fact, a typical "stroma reaction" occurs. This consists in the accumulation of extracellular material and connectival cells: in this process myofibrablasts are involved. It is known that the fibroblasts recruited inside the tumor mass have different metabolic features with respect to the "normal" ones. They actively stimulate the tumor mass growth and effectively induce its vascularization (7-12). A new class of protein kinase inhibitors has been recently identified. Their chemical basis is the 6-aryl-pyrido[2,3-d]pyrimidine (13). Chemical substitutions of this fundamental structure led to the synthesis of the pyrido[2,3]pyrimidine (Fig.1) also known as PD166866. This is a very selective inhibitor of FGFr 1 (14). The drug presents several features which differentiate it from the "classical" protein kinase inhibitors, in particular: a) it is the most potent inhibitor of the FGFr 1 tyrosine kinase at nanomolar concentrations; b) it competes with ATP for FGFr 1 binding to tyrosine kinase; c) it does not exert any inhibitory effect on several other receptors such as c-Src, EGFr and the insulin receptor; d) it inhibits the auto-phosphorilation of the FGF receptor mediated by bFGF; e) it inhibits the phosphorylation of bFGF induced by the MAP kinase. One of the research project developed in our laboratory is focused on the cell stress response and on the evaluation of antiviral and antiproliferative properties of natural and synthetic substances (15-19).

On the basis of the literature data discussed above it

is possible to hypothesize the use PD166866 as antiproliferative and/or antiangiogenic agent to counteract uncontrolled proliferation and neo-angiogenesis in cancer. In perspective an evaluation of the biological action of this drug is, therefore, an essential prerequisite for its potential use in clinical applications. This work is aimed at the elucidation of the cytotoxic potential in a model of cultured murine fibroblasts.

Materials and Methods

Drug synthesis

PD 166866 was prepared according to the standard literature method (20).The structure was determined and is shown in Fig. 1. The drug was dissolved in dimethylsulphoxide (DMSO) prior to use in 10 mM stock solutions.

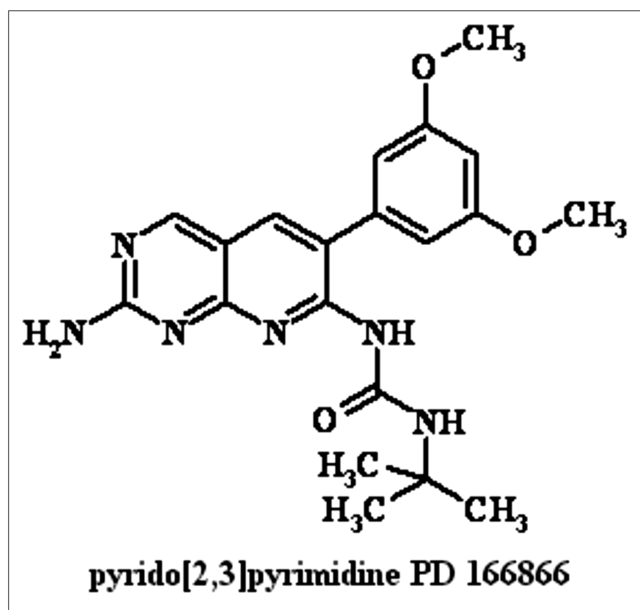

Fig. 1 - Structure formula of the pyrido[2,3]pyrimidine PD 166866.

Cell line and maintenance

The stable cell line of murine fibroblasts 3T6 was used; cultures were routinely cultured at 37°C in 5% carbon dioxide atmosphere in DMEM supplemented with 10% newborn serum, glutamine (50 mM f.c.) and penicillin-streptomycin (10000 U/ml each). Cell cultures were split every second day and never passaged more than 15 to 20 times.

Cell viability assessment

After 48 hrs of exposure to PD166866, viable cells were assessed by the MTT assay (21). The amount of formazan blue crystal formation was measured at 570 nm. The actual number of viable cells was estimated by comparison with standard calibration curve correlating between absorbance and actual cell number. Results of the experiments with MTT were analyzed by one way ANOVA and Multiple Range Tests LSD with significance level 0.05 using SPSS for Windows (22). Measurements were replicated at least three times.

Immunofluorescent TUNEL staining

Cells ($7x10^4$) were grown on 30 mm Petri dishes for 48 hrs and treatment with PD168688 was performed as above. To permeabilize cell nuclei 0,1% Triton X-100 was used in a 3.4 mM sodium citrate aqueous solution. TUNEL staining was carried out by a commercial kit ("*In situ*" Cell Death Detection Kit", Roche Diagnostic).

Immunolocalizations of Proliferating Cell Nuclear Antigen (PCNA)

Treated cells were fixed in 4% paraformaldehyde, washed in PBS and permeabilized with Buffer P (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Immunolocalizations were performed using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. PCNA (mouse, anti-PCNA, IgG2a, kappa, Dako) assay was performed as described previously (18). Samples were observed by optical microscope

Results

PD166866 has a cytotoxic effect

The cytoxicity of PD168688 was evaluated after 48 hrs of treatment at a final concentration of $0,5x10^{-4}$ and $1.0x10^{-4}$ M. An evident reduction of viability, measured by MTT is shown Figure 2. The drug was dissolved in DMSO and a final concentration of 0.5% v/v. The same amount of this compound was present in control plates where no reduction of vital cell number was observed (not shown). Therefore the loss of viability is attributable solely to PD166866. Serum deprivation has a slightly lower lethal effect than treatment with the drug.

TUNEL staining demonstrates cell death in treated cells

Extensive cell death is caused by treatment with

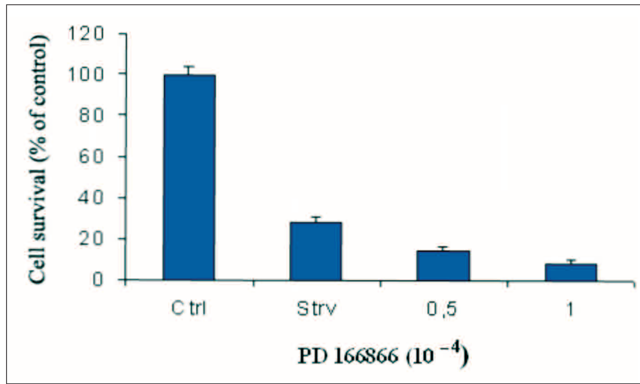

Fig. 2 - Vital cell counts in control and PD166866 treated cells.

PD166866 as evidenced by TUNEL staining (Fig. 3, Panel C). Also in this case a more relevant fluorescent staining is observed after treatment with the drug as compared to serum deprivation. Therefore this data are consistent with the one obtained in the previous experiment.

Reduction of PCNA after drug treatment

The result in Figure 4 clearly shows a drastic reduction of expression of the proliferating cell nuclear antigen (Panel C). An overall decrease of cell number is also evident in the micrograph. This result further supports the idea that PD166866 exerts an antiproliferative action.

Discussion

Fibroblasts play an important role in a variety of biological processes. They are involved in the formation and organization of the extra-cellular matrix, in the production of growth factors as well as cytokines and

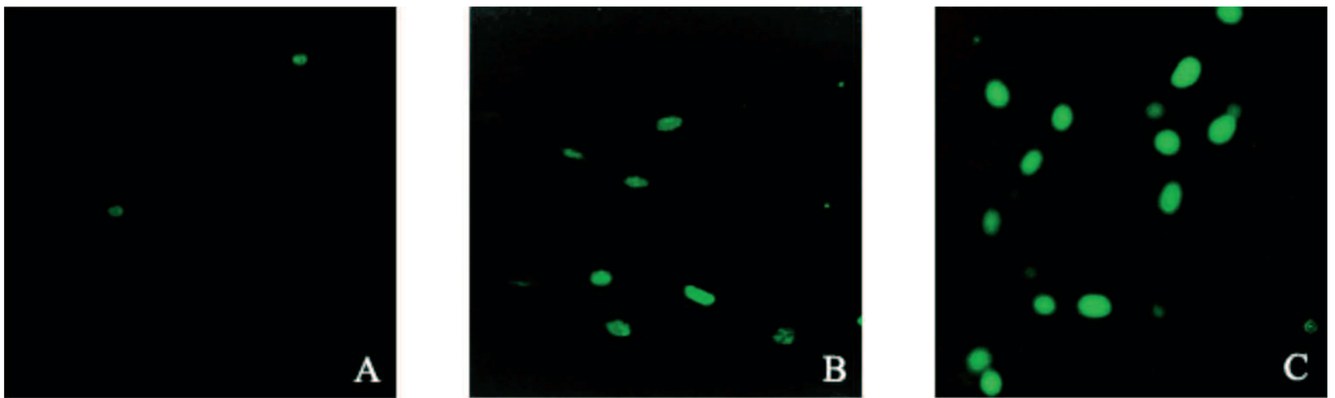

Fig. 3 - Tunel staining of control (A), serum deprived (B) and PD166866 treated cells (C). Only the effect of 0.5 x 10^{-5} M drug is shown since this is the minimal concentration require to monitor the cytotoxic effect.

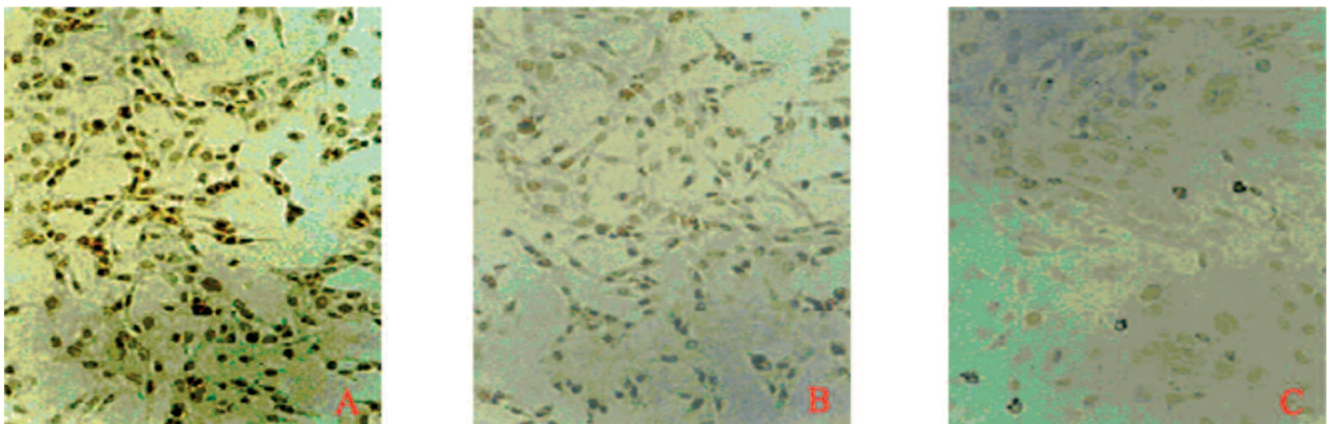

Fig. 4 - Immunolocalization of PCNA in control (A), serum deprived (B) and PD166866 treated cells (C). Drug concentration was 0.5 x 10^{-5} M.

chemokines. Their production of paracrine modulators makes them play a key role in tumor development and progression. Cultured fibroblasts, furthermore, are a good model system to investigate a number of different phenomena at biomolecular/cellular level. In our laboratory, cultured mouse fibroblasts (3T6 cells) were used to investigate the molecular action of natural substances such as monensin and usnic acid (15,16). In this work we report on the role of a synthetic drug known as PD166866 in cell proliferation control. This compound was also shown to be a potent inhibitor of FGFR-1 tyrosine kinase and to exert a selective action on a variety of receptors such as PDGFR, EGFR, c-src and the insulin stimulated receptor tyrosine kinases (14). Its inhibition of growth factors led to assume that administration of PD166866 to cultured cell might be a good tool to control cell proliferation. Exposure of cells to the drug actually does cause a significant reduction of cell growth already at micro-molar concentrations as shown by vital cell counts. The significantly higher fluorescence monitored in treated cells shows that the drug exerts a cytotoxic effect. Since positivity to TUNEL reaction is generally assumed as an indication of apoptosis, these data imply that cell death might occur via an apoptotic pathway as expected from growth factor depletion. As a matter of fact these factors are required for cell factor survival and proliferation. This hypothesis, however, requires further deepening. In any case, the decrease of intranuclear levels of Proliferating Cell Nuclear Antigen (PCNA) is suggestive of an impaired DNA replication. Interestingly, PD166866 seems to induce cell death more efficiently than serum deprivation, a commonly accepted way to induce cell death by oxidative stress. It is worth noting that the phenomenon was observed both by vital cell count and TUNEL staining: two completely diverse techniques, aimed at the evaluation of two different aspects of cell survival.

In conclusion, the results presented in this short communication strongly suggest that PD166866 might be used in the control of hyper-proliferative fibrotic pathologies like tumors. Further studies are necessary to evaluate possible clinical and pharmacological applications in humans.

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