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Poly(ADP-ribose) polymerase (PARP) inhibition or PARP-1 gene deletion reduces angiogenesis

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ABSTRACT

Poly(ADP-ribose) polymerase (PARP)-1 has recently been shown to promote tumour progression. Since angiogenesis is an essential requirement for tumour growth, we examined whether PARP inhibition/deletion might affect endothelial cell functions. To this end, the influence of PARP inhibitors on endothelial cell proliferation, migration, tube formation and angiogenesis in PARP-1 knock-out mice, using an *in vivo* matrigel plug assay, was investigated.

The results indicated that the PARP inhibitor GPI 15427 (IC_{50} on endothelial PARP: 237 ± 27 nM), at concentrations devoid of cytotoxic effects (0.5–1 μM), abrogated migration in response to vascular endothelial growth factor or placenta growth factor, hampered formation of tubule-like networks and impaired angiogenesis *in vivo*. The anti-angiogenic effect of the PARP inhibitor was confirmed in PARP-1 knock-out mice that displayed a defect of angiogenesis induced by growth factors.

These results provide evidence for targeting PARP for anti-angiogenesis, adding novel therapeutic implications to the use of PARP inhibitors in cancer treatment.

1. Introduction

Poly(ADP-ribosy)lation, a post-translational modification of proteins, plays a crucial role in many biological processes. The best characterised poly(ADP-ribosyl)ating enzyme, PARP-1, is involved in a number of cellular functions, including the maintenance of genomic integrity, DNA repair, apoptosis and regulation of gene expression.[1–3] The enzyme becomes rapidly activated upon DNA damage and, after binding to DNA strand breaks, forms homodimers and catalyses the synthesis of ADP-ribose polymers from NAD^+ onto itself and other nuclear proteins. Poly(ADP-ribose) is then quickly degraded by poly(ADP-ribose) glycohydrolase (PARG).

PARP-1 and its closest analog PARP-2 are the components of the base excision repair (BER) system. Both are involved

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in the repair of *N*-methylpurines, which are generated by DNA methylating agents such as temozolomide.[4–7] Moreover, PARP-1 seems to have a key role in the repair of double-strand breaks produced by ionising radiations or cisplatin.[8,9] Poly-(ADP-ribos)ylated PARP-1/-2 counteracts the action of topoisomerase I poisons by facilitating resealing of DNA strand breaks.[10] Therefore, PARP inhibition has been explored to enhance the efficacy of chemo- or radiotherapy for cancer treatment and a number of PARP inhibitors are presently in Phase I/II clinical trials.[11,12] In addition to the potentiation of chemo- and radio-therapies, recent reports have shown that PARP inhibition alone selectively kills tumour cells carrying mutations in the breast cancer associated genes 1 and 2 (BRCA-1 or BRCA-2) or in other genes involved in the homologous recombination (HR) repair machinery.[13–15]

It is well characterised that the growth of tumours strictly depends on the establishment of a microcirculation in order to grow beyond a few millimetres and that metastatic cells are only shed after the tumour establishes its microcirculation. The intrusion of a vascular network requires different sequential steps including the release of proteases from 'activated' endothelial cells with subsequent degradation of the basement membrane surrounding the existing vessel, migration of endothelial cells into the interstitial space, endothelial cell proliferation and differentiation into mature blood vessels. These processes are mediated by a wide range of angiogenic factors, including growth factors, pro-inflammatory cytokines, chemokines, angiogenic enzymes, endothelial receptors and adhesion molecules. Therefore, a number of different approaches targeting angiogenic factors, antagonising their binding to receptors, and interrupting signal transduction, have been extensively investigated for anticancer therapy.[16]

The process of neovascularisation is regulated by numerous factors and matrix proteins released from host stromal cells, such as macrophages, lymphocytes and fibroblasts. Leukocytes compose a large percentage of the total cellular repertoire in many tumour types. Thus, the inflammatory component of a developing neoplasm is remarkable.[17]

The major role of PARP-1 in inflammatory diseases and the protective effect of PARP inhibitors have been demonstrated in various experimental models.[18] Besides the involvement in cell death pathways due to extensive NAD^+ consumption during the synthesis of (ADP-ribose) polymers, which leads to ATP depletion and severe energetic crisis of the cell, PARP-1 enhances the activities of key transcription factors regulating the expression of inflammatory mediators and adhesion molecules. Interestingly, it has been recently demonstrated that the inhibition of PARP prevents skin carcinogenesis through its ability to regulate the transcription of genes involved in inflammation.[19]

Recently, it has been reported that pharmacological PARP inhibition reduces angiogenesis in *in vitro* and ex vivo models.[20,21] In the present study, we demonstrate an impairment of blood vessel neo-formation in response to angiogenic stimuli *in vivo* using a PARP inhibitor, previously reported to enhance the efficacy of antitumour agents. The involvement of PARP-1 in angiogenesis has been demonstrated in knockout mice that displayed a defect of angiogenesis induced by growth factors.

2. Materials and methods

2.1. Cell culture and treatment with PARP inhibitor

The immortalised human endothelial cell line HUV-ST was generated as previously described.[22] Cells were maintained in culture in endothelial growth factor medium (EGM-2; Clonetics, BioWhittaker Inc, Walkersville, MD) supplemented with 0.4 mg/ml geneticin and 5 μg/ml puromycin. Human umbilical vein endothelial cells (HUVEC), isolated from freshly delivered umbilical cords, were kindly provided by Dr. Orecchia (IDI-IRCCS, Rome, Italy) and cultured in EGM-2.

For PARP inhibition in endothelial cells, the recently developed compound GPI 15427 (10-(4-methyl-piperazin-1-ylmethyl)-2*H*-7-oxa-1,2-diaza-benzo[*de*]anthracen-3-one, MGI Pharma, Baltimore, MD) or, in a selected experiment, 4 mM 3-aminobenzamide (AB, Sigma-Aldrich, St. Louis, MO) were used.

2.2. Analysis of cell growth

Cell proliferation was evaluated using a Promega kit, according to the manufacturer's instructions (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI). The assay utilises the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulphophenyl)-2*H*-tetrazolium, inner salt (MTS). Long-term cell survival was determined by means of colony-forming assay.

2.3. Cell adhesion assays

Solid support was prepared by coating immunological 96-multiwell plates with 100 μg/ml matrigel (BD Biosciences), 10 μg/ml fibronectin, laminin, vitronectin (Sigma–Aldrich) or collagen IV (BD Biosciences) in PBS for 18 h, and blocking with 3% (w/v) BSA in PBS for 2 h. Untreated endothelial cells or cells (4×10^4/well) exposed to PARP inhibitor were plated in serum-free medium supplemented with 0.1% (w/v) BSA. After incubation at 37 °C for 45 min, attached cells were fixed with 3% (v/v) formaldehyde and stained with 0.5% (w/v) crystal violet. The attachment efficiency was determined by quantitative dye extraction and spectrophotometric measurement of the absorbance at 595 nm.

2.4. Migration assay

The migration assay was performed in Boyden chambers endowed with polycarbonate filters (Nucleopore, Whatman incorporated, Clifton, NJ) coated with gelatine, as previously described.[22] The following stimuli for chemotaxis were used: 50–100 ng/ml of vascular endothelial growth factor A (VEGF), 50 ng/ml placental growth factor (PlGF) or 100 ng/ml epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN). Cells (1.5×10^5) were loaded into each chamber and the effect of GPI 15427 on cell migration was analysed by incubating the cells with the drug 1 h before performing the assay. Background migration was evaluated by including basal medium (0.1% (w/v) BSA in endothelial basal medium (EBM-2)) instead of the stimulus. After 18 h (for HUV-ST cells) or 5 h (for

HUVEC) incubation at 37 °C, the filter was removed from the camera.

The chemotactic response was determined by counting the migrating cells attached to the lower surface of the filter in 12 randomly selected microscopic fields (×200 magnification). The results were expressed as chemotactic index (i.e. the ratio between the number of cells/field in the samples stimulated by the growth factor and the number of cells/field in the absence of the stimulus).

2.5. Matrigel angiogenesis assay in vitro

The effect of GPI 15427 on endothelial cell differentiation was analysed by incubating the cells with different drug concentrations (0.1, 0.5 or 1 μM) 1 h before layering the cells onto the matrigel, as previously described.[22,23] Endothelial cell alignment was quantified by counting the intersections of the newly formed capillary-like structures.

2.6. Western blot analysis

For the analysis of hypoxia inducible factor-1α (HIF-1α) induction, HUV-ST cells, untreated or pre-incubated for 1 h with 1 μM GPI 15427, were exposed for 6 h to the hypoxia mimetic agent $CoCl_2$ (150 μM). For the analysis of FAK and ERK1/2 phosphorylations, HUV-ST cells were starved for 24 h in 0.1% (w/v) BSA/EBM-2 supplemented with glutamine and gentamicin before starting the experiment. Cells were then treated for 1 h with increasing concentrations of GPI 15427 (0.1–2 μM) and afterwards stimulated for 10 min with 100 ng/ml of VEGF or PlGF in the same medium supplemented with 1 μg/ml heparin. After cell lysis, equal amount of proteins were run in a 10% (w/v) SDS–polyacrylamide gel and then transferred to nitrocellulose membranes. The following antibodies were used: anti-FAK(pY397), anti-ERK1/2(pTpY185/187) (Biosource, Camarillo, CA, 1/1000 dilution), anti-HIF-1α (R&D Systems, 1/200 dilution) and anti-tubulin (Santa Cruz, Santa Cruz, CA, 1/1000 dilution). Detection was carried out using the ECL western blotting detection reagents (GE Healthcare, Milan, Italy).

2.7. Matrigel angiogenesis assay in vivo

Eight week-old C57BL/6 (Charles River, Calco, Italy) or 129sv WT or PARP-1 KO mice[24] were injected subcutaneously (s.c.) in the flank with 600 μl of matrigel (BD Biosciences) supplemented with VEGF (100 ng/ml) or PlGF (100 ng/ml) (R&D Systems) and heparin (19U; Schwarz Pharma SpA, Milan, Italy). In selected cases, GPI 15427 was added to matrigel at a final concentration of 4 μg/ml, corresponding to the plasma peak concentration of the PARP inhibitor reached after a single intravenous dose of 40 mg/kg in rats.[6] Negative controls contained heparin alone. Each group consisted of six animals. After 5 days mice were sacrificed and matrigel plugs were harvested. The angiogenic response was evaluated by macroscopic analysis of the plug at autopsy and by measurement of the haemoglobin (Hb) content into the pellet of matrigel. Hb was mechanically extracted from the pellets in water and measured using the Drabkin (Sigma–Aldrich) method by spectrophotometric analysis at 540 nm. Values were expressed as optical density (OD)/100 mg of matrigel. Matrigel plugs were fixed in formalin, included in paraffin and stained by haematoxylin and eosin (H&E). Morphology and evaluation of blood vessel density were analysed by optical microscopy.

2.8. PARP activity assay

HUV-ST cells (5×10^6) were lysed in 0.5 ml of a buffer containing 0.1% (v/v) Triton-X, 50 mM Tris–HCl, pH 8, 0.6 mM EDTA, 14 mM β-mercaptoethanol, 10 mM $MgCl_2$ and protease inhibitors. Proteins (25 μg) were incubated with 2 μCi ^{32}P-NAD$^+$ (GE Healthcare, Milan, Italy), 10 μM NAD$^+$, 50 mM Tris–HCl, 10 mM $MgCl_2$, 14 mM β-mercaptoethanol and 10 μg nuclease-treated salmon testes DNA. PARP activity was expressed as fmol of ^{32}P-NAD$^+$/μg of protein.[5]

For the analysis of the influence of GPI 15427 on cellular PARP, intact HUV-ST cells (5×10^5 cells) were treated with the inhibitor and permeabilised with digitonin (0.1 mg/ml) in the presence of 0.25 μCi ^{3}H-NAD$^+$ (Perkin–Elmer, Milan, Italy).[7] For PARP-2 inhibition by GPI 15427 (3–300 nM), the IC_{50} was determined using 100 ng of purified recombinant murine PARP-2 (Alexis Italia, Florence, Italy).

For the analysis of PARP activity in white blood cells (WBC) of 129sv WT or PARP-1 KO mice, 300 μl of peripheral blood was collected from the retro-orbital sinus. After the removal of red blood cells by red blood lysing buffer (Sigma–Aldrich), WBCs were exposed to 20 mM H_2O_2 for 15 min, permeabilised with digitonin and incubated with ^{3}H-NAD$^+$.

3. Results

3.1. The PARP inhibitor GPI 15427 inhibits PARP activity at concentrations that do not affect endothelial cell proliferation

An immortalised human endothelial cell line generated in our laboratory from HUVEC has been used to evaluate the effects of PARP inhibitor on migration and capillary-like networks formation.[22] This cell line (HUV-ST) possesses stabilised telomere length and increased proliferation rate with respect to parental cells. Interestingly, HUV-ST cells over-express the tumour endothelial marker TEM-1, which is regarded as the most differentially expressed molecule in tumour-derived endothelium versus normal-derived endothelium. Nevertheless, it is not tumourigenic and displays all major endothelial phenotypic markers, such as von Willebrand factor, CD31, CD105/endoglin and VEGF receptors (VEGFR1/Flt-1, VEGR2/KDR). Like HUVEC, HUV-ST cells are capable of organising into tubule-like networks with branching morphology in response to appropriate stimuli and migrate upon exposure to angiogenic factors such as VEGF, PlGF or EGF. Therefore, the immortalised human endothelial line HUV-ST represents a suitable model for studying the efficacy of anti-neovascular therapy, mimicking proliferating neovascular endothelial cells associated to the tumour mass.

GPI 15427 was used to inhibit PARP activities. This compound is highly potent in inhibiting both PARP-1[5–7] and PARP-2 activities (IC_{50}: 8 ± 2 nM) and has been shown to act as chemosensitizer in *in vitro* and *in vivo* preclinical models.[5–7] Initially, HUV-ST cells were exposed to graded concentrations

of the PARP inhibitor GPI 15427 (1–100 μM) and cell growth was analysed 3 and 5 days after treatment by MTS assay or after 10 days by colony formation assay. The results of MTS assay indicated that in endothelial cells GPI 15427 displays antiproliferative effects with an IC_{50} of 25 ± 3 μM at 3 days of culture or 17 ± 3 μM at 5 days. Finally, GPI 15427 IC_{50} evaluated by colony formation assay was 21 ± 2 μM.

HUV-ST cells were then analysed for PARP activity, measured in cell extracts in the presence of nuclease-treated salmon testes DNA and ^{32}P-NAD$^+$. The results indicated that total PARP activity of HUV-ST cells was 853 ± 178 fmol/μg of protein. We then tested the ability of GPI 15427 to inhibit PARP activity of intact endothelial cells by the exposure of HUV-ST cells for 1 h to graded concentrations of GPI 15427 (0.1–2 μM), followed by permeabilisation with digitonin in the presence of ^{3}H-NAD$^+$. The results indicated that GPI 15427 easily penetrated into the cells and inhibited PARP activity with an IC_{50} of 237 ± 27 nM.

3.2. *GPI 15427 impairs tube-like structures formation in matrigel and inhibits endothelial cell migration in response to PlGF and VEGF*

To investigate the ability of PARP inhibitor to modulate angiogenesis in vitro, HUV-ST cells were exposed to GPI 15427 concentrations (0.1–1 μM) devoid of antiproliferative effects and

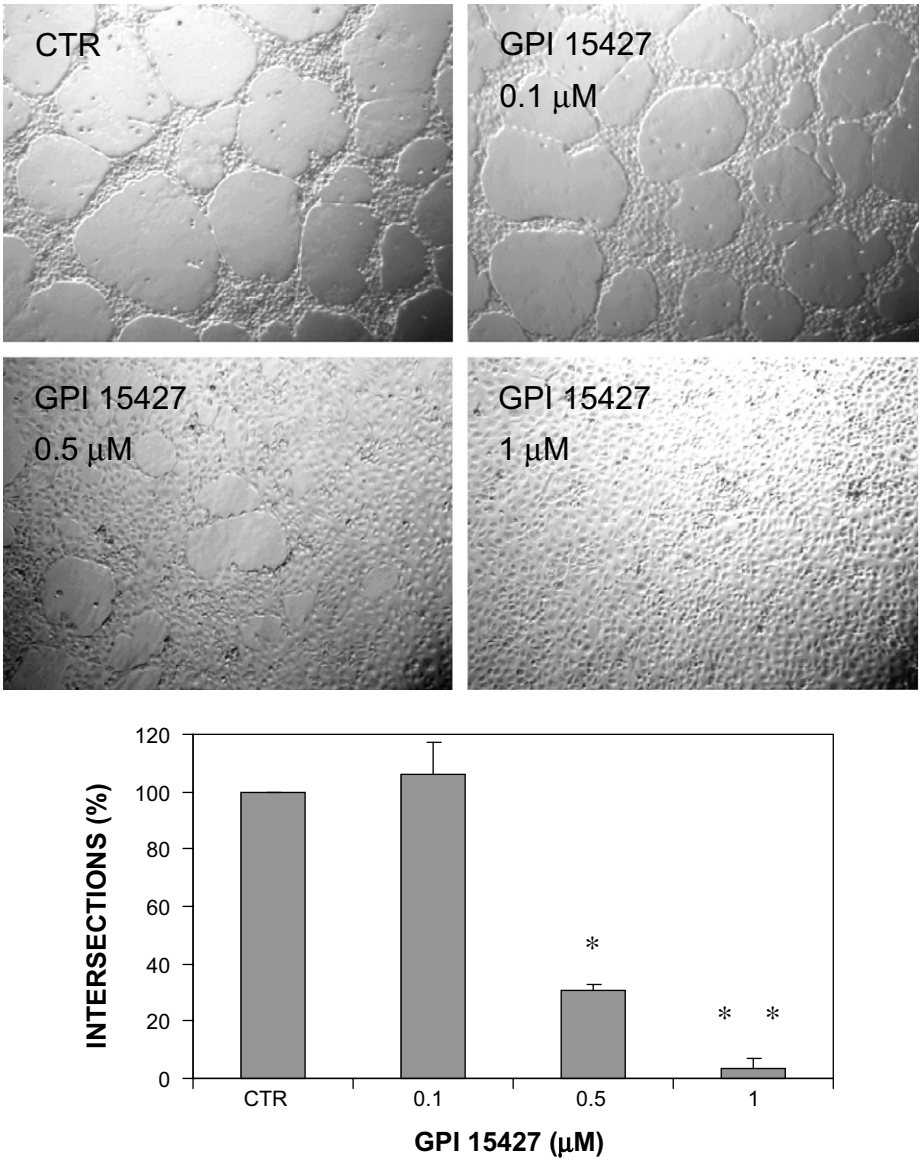

Fig. 1 – GPI 15427 inhibits tube-like structures formation by endothelial cells. HUV-ST cells were grown on matrigel in the presence of the indicated concentrations of GPI 15427 and tube formation was analysed after 16 h. Phase contrast, × 50 magnification. The number of intersections of the capillary-like structures formed was counted in three different microscopic fields and the mean percentage of intersections with respect to the untreated control was calculated. Histograms and bars represent mean (calculated following angular transformation of the percentage values) + standard deviation (SD). Statistical analysis (Student's t-test) indicated that the differences between untreated group and groups treated with 0.5 and 1 μM were statistically significant [*P = 0.0003; **P < 0.0001, respectively].

then allowed to form tube-like structures in matrigel. The results, illustrated in Fig. 1, indicated that 0.5 and 1 μM GPI 15427 markedly inhibited and abrogated tube formation, respectively. This effect was not due to a direct cytotoxic effect of the drug, since cells treated with 1 μM GPI 15427 were viable and capable of growing in monolayer (Fig. 1).

The ability of GPI 15427 to modulate endothelial cell chemotaxis in response to various stimuli was then analysed in an *in vitro* migration assay performed in Boyden chambers. Proliferating HUV-ST cells or primary HUVEC were harvested and treated with 0.5 μM GPI 15427 for 1 h. Cells were then loaded into the chambers and allowed to migrate towards different stimuli. The results, illustrated in Fig. 2, show that in both cell cultures GPI 15427 completely inhibited endothelial cell migration in response to PlGF or VEGF, while induced a negligible reduction of the chemotactic response to EGF. When cells were treated with the weaker PARP inhibitor AB (4 mM) abrogation of migration in response to PlGF and VEGF was also observed (data not shown).

The possibility that GPI 15427 could affect endothelial cell functionality by altering cell adhesion to matrigel or to single components of the extracellular matrix, such as fibronectin, laminin, collagen IV or vitronectin was also analysed in cell adhesion assays. Tests were performed on cell culture plates coated with matrigel or with the different extracellular matrix components, after the incubation of the cells for 1 h with GPI 15427 (from 0.1 to 1 μM). No difference in adhesion ability was observed when untreated HUV-ST cells were compared with cells exposed to the drug (data not shown).

In view of the role of hypoxia-inducible factor-1α (HIF-1α) in angiogenesis and of the ability of a different PARP inhibitor to counteract activation of the transcription factor in fibroblasts,[19] we have investigated whether the exposure of endothelial cells to 1 μM GPI 15427, a concentration capable of inhibiting tube formation and migration, might affect HIF-1α activation induced by the hypoxia mimetic agent $CoCl_2$. The results show that GPI 15427 did not inhibit HIF-1α induction by $CoCl_2$ (Fig. 3).

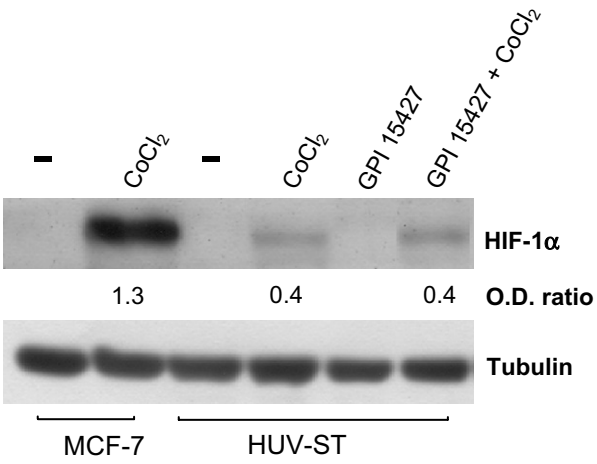

Fig. 3 – Treatment with GPI 15427 does not affect the activation of HIF-1α by hypoxia mimetic stimulus in endothelial cells. HUV-ST cells, untreated or pre-incubated for 1 h with 1 μM GPI 15427, were exposed for 6 h to the hypoxia mimetic agent $CoCl_2$ and then processed for immunoblot analysis. Human breast cancer MCF-7 cells treated with $CoCl_2$ for 6 h were used as positive control for HIF-1α induction, according to R&D Systems instructions. Numbers indicate the ratios between optical densities of HIF-1α and those of tubulin of cells treated with $CoCl_2$ or treated with $CoCl_2$ in the presence of GPI 15427.

3.3. *GPI 15427 inhibited in vivo angiogenesis in the matrigel plug assay*

The effect of the PARP inhibitor on *in vivo* angiogenesis was evaluated using the matrigel plug assay. Macroscopic analysis

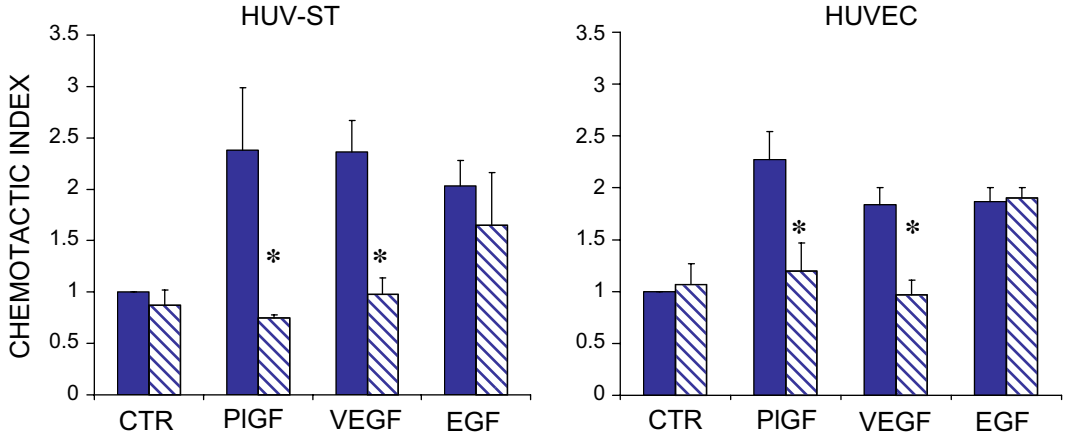

Fig. 2 – Effect of GPI 15427 on endothelial cell migration in response to PlGF, VEGF or EGF. The effect of GPI 15427 (0.5 μM) on the migration of HUV-ST cells or primary HUVEC induced by VEGF, PlGF or EGF was evaluated in Boyden chambers. Histograms represent the mean of the chemotactic indexes in response to PlGF, VEGF or EGF for three independent experiments, and bars represent the SD. For both immortalised and primary endothelial cells statistical analysis (Student's *t*-test) of the differences between untreated groups (solid bars) and groups treated with GPI 15427 (hatched bars) was as follows: CTR (absence of chemotactic stimulus) not significant; VEGF, $P < 0.0001$ (*); PlGF, $P < 0.0001$ (*); EGF, not significant.

of matrigel plugs containing heparin and VEGF injected s.c. in the flank of C57BL/6 mice revealed an intense vascularisation, whereas only a slight vessel formation was observed when GPI 15427 was added to the matrigel plugs containing the pro-angiogenic factors (Fig. 4). The angiogenic response observed by macroscopic analysis was confirmed by the quantitative measurement of the Hb content of the excised matrigel plugs (Fig. 4). Hb levels detected in GPI 15427 treated plugs were significantly lower than those measured in positive control ($P < 0.0001$). Similar results were obtained when PlGF was used as pro-angiogenic factor (data not shown; GPI 15427 *versus* positive control, $P < 0.0001$). Histological examination of matrigel plugs containing heparin and VEGF indicated the presence, throughout the section, of vascular structures endowed with lumen and circulating red blood cells (Fig. 4, panels A and B); whereas plugs containing only heparin without the growth factor were colonised by elongated single cells not forming cord-like structures (Fig. 4, panels C and D). Matrigel containing heparin, VEGF and GPI 15427, was mostly devoid of any colonisation (Fig. 4, panels E and F).

3.4. *Reduction of in vivo angiogenesis in PARP-1 KO mice*

To confirm the involvement of PARP-1 in angiogenesis triggered by VEGF or PlGF, the matrigel plug assay was performed in WT or PARP-1 KO sv129 mice. Mice were initially analysed for PARP-1 proficiency or deficiency by testing their cellular response to the oxidant H_2O_2 that is known to generate DNA strand breaks through the formation of hydroxyl radicals and to induce PARP-1 activation. The results of PCR analysis of genomic DNA showed the amplification of the 1.2 kb intact allele and 1.4 kb KO allele in WT and PARP-1 deficient mice, respectively (data not shown). Moreover, the results of PARP activity assay in permeabilised WBC collected from peripheral

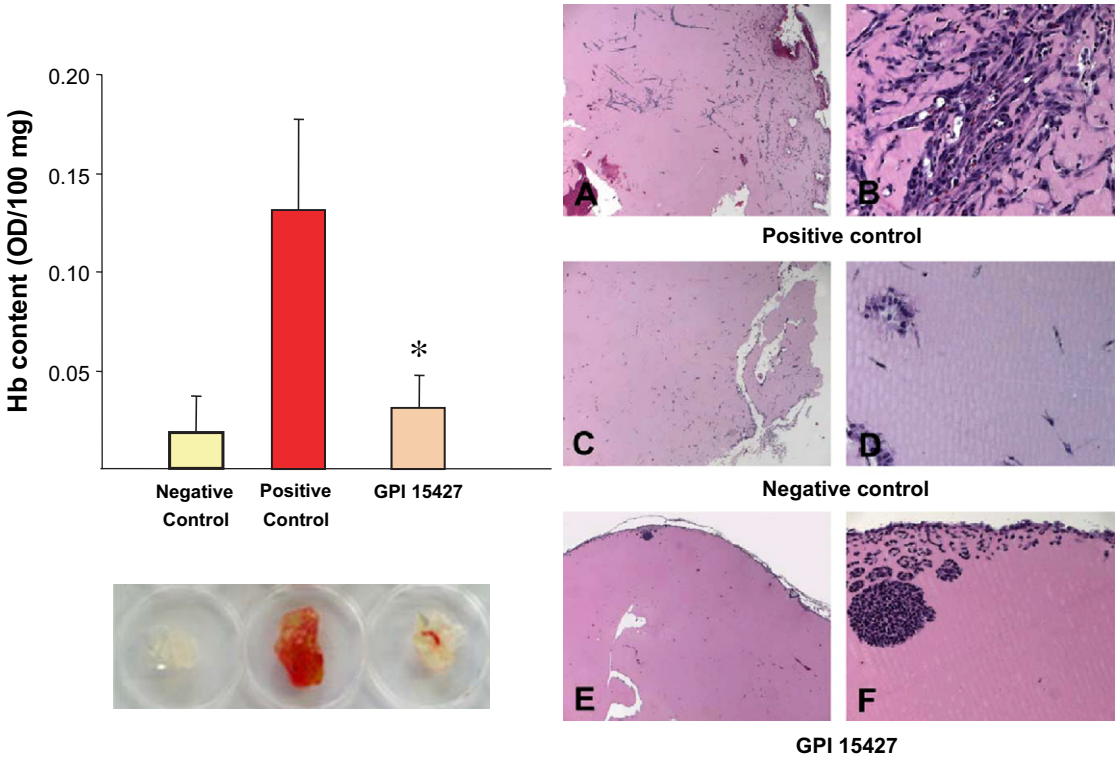

Fig. 4 – GPI 15427 inhibits *in vivo* angiogenesis evaluated by matrigel plug assay. *Left panel*: *In vivo* vessel formation was assessed after the injection of C57BL/6 mice with matrigel plugs containing heparin and VEGF (positive control) or containing heparin only (negative control). C57BL/6 mice were also injected with plugs containing heparin, VEGF and GPI 15427 (4 μg/ml). After 5 days, animals were sacrificed, and neovascularisation was evaluated by macroscopic analysis and by the measurement of Hb content of matrigel plugs. The macroscopic appearance of representative matrigel plugs from each experimental group is shown. Histograms represent the mean value ($n = 12$; data from two independent experiments) of the Hb content, expressed as absorbance (OD)/100 mg of matrigel plug. Bars: +SD values. GPI 15427 *versus* positive control: $P < 0.0001$ (*) (Student's *t*-test); GPI 15427 *versus* negative control: not significant. Right panel: morphological evaluation of neoangiogenesis in matrigel's subcutaneous implants. Matrigel plugs were fixed in formalin, included in paraffin and H&E stained. Positive control: (A) cord like colonisation is evident throughout the section. Lakes of red blood cell are present at the periphery of the matrigel pellet (25×). (B) At a larger view many of the cord-like structures show a lumen with circulating blood cells (250×). Negative control: (C) The matrigel is colonised by elongated single cells, occasionally forming cord like structures at the pellet periphery, where no lakes of red blood cells could be seen (25×). (D) The peripheral colonising cells may appear organised in incomplete rosettes (250×). GPI 15427: (E) The matrigel is mostly devoid of any colonisation; occasionally, small clusters of cells are seen at the periphery (25×). (F) The clusters are composed of small round cell organised in rosettes (100×).

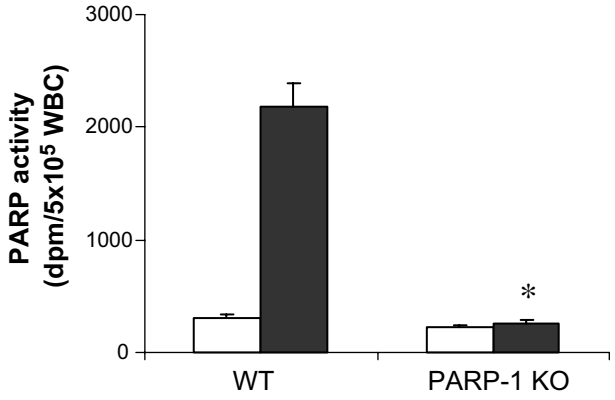

Fig. 5 – Lack of PARP-1 activation by genotoxic damage in PARP-1 KO mice. Analysis of PARP activation in WBC collected from WT ($n = 3$) or PARP-1 KO ($n = 3$) mice was assessed by incubating cells, untreated (open bars) or exposed (solid bars) to 20 mM H_2O_2 for 15 min, with ^{3}H-NAD$^+$ after permeabilisation with digitonin. The results are expressed as dpm of trichloroacetic acid-precipitable ^{3}H-NAD$^+$/5 × 10^5 cells. Bars: +SD values. H_2O_2 treated WBC from WT mice versus H_2O_2 treated WBC from PARP-1 KO mice: $P < 0.0001$ (*) (Student's t-test).

blood revealed the lack of PARP activation upon H_2O_2 exposure in the cells collected from PARP-1 KO mice (Fig. 5).

Macroscopic analysis of the matrigel plugs containing heparin and VEGF or the determination of Hb levels indicated a reduction of vessel formation in PARP-1 KO mice with respect to WT sv129 mice ($P = 0.0001$) (Fig. 6). The extent of reduction of vessel growth in matrigel plugs in PARP-1 KO (Fig. 6) was similar to that observed in C57BL/6 mice when GPI 15427 was included in the matrigel plug (Fig. 4). Comparable results were obtained when PARP-1 KO mice were injected with matrigel plugs containing PlGF as angiogenic stimulus in place of VEGF (PARP-1 KO versus WT mice, $P = 0.007$) (Fig. 7).

Finally, in WT sv129 mice the addition of GPI 15427 to the matrigel plug induced an ~80% reduction of vessel formation and in PARP-1 KO mice it abrogated the angiogenic process (data not shown, $P < 0.0001$).

4. Discussion

In the present study, we demonstrate for the first time that the impairment of PARP-1 function hampers angiogenesis as indicated by the reduction of blood vessel neo-formation in response to angiogenic stimuli observed in PARP-1 KO mice or in endothelial cells treated with GPI 15427, a recently developed PARP inhibitor, previously reported to enhance the efficacy of antitumour agents.[5–7]

The *in vitro* studies were conducted using an immortalised cell line, which represents a suitable model for studying the efficacy of anti-neovascular therapy, mimicking proliferating

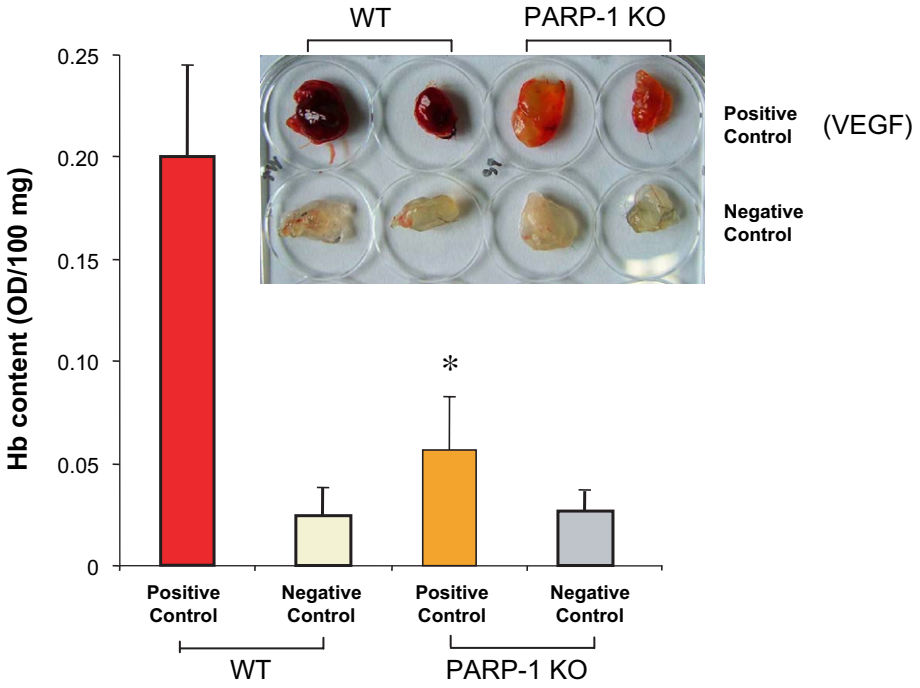

Fig. 6 – Reduction of *in vivo* angiogenesis triggered by VEGF in PARP-1 KO mice. *In vivo* vessel formation was assessed after injection of WT and PARP-1 KO sv129 mice with matrigel plugs containing VEGF and heparin (positive control) or containing heparin only (negative control). After 5 days, animals were sacrificed, and neovascularisation was evaluated by macroscopic analysis and measurement of Hb content of matrigel plugs. The macroscopic appearance of typical plugs from each experimental group is shown. Histograms represent the mean value ($n = 12$; data from two independent experiments) of the Hb content, expressed as OD/100 mg of matrigel plug. Bars: +SD values. Positive control of PARP-1 KO mice versus positive control of WT mice: $P = 0.0001$ (*) (Student's t-test).

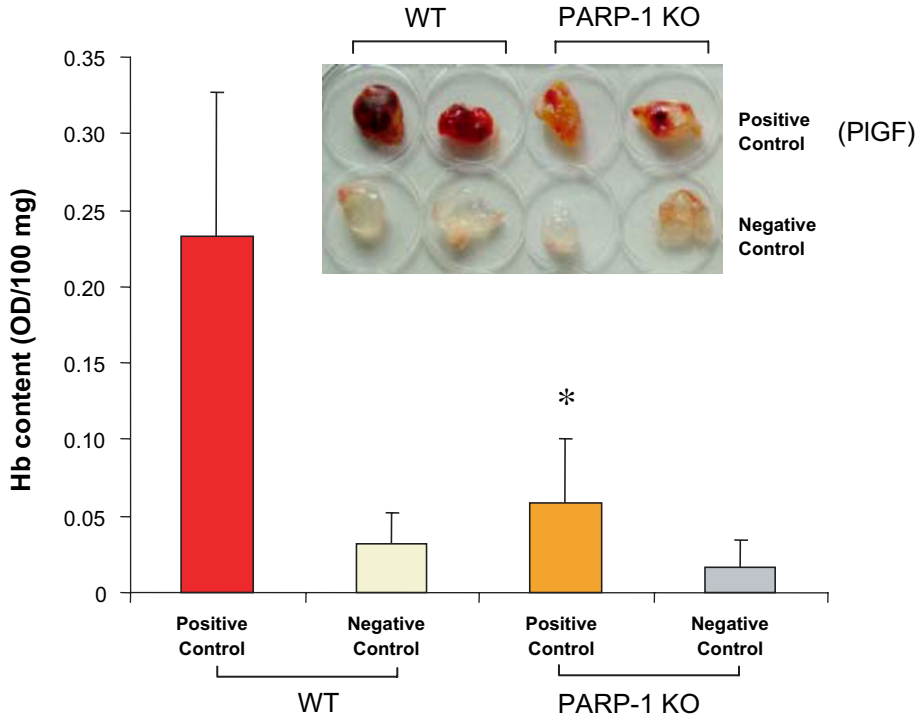

Fig. 7 – Reduction of *in vivo* angiogenesis triggered by PlGF in PARP-1 KO mice. *In vivo* vessel formation was assessed after the injection of WT and PARP-1 KO sv129 mice with matrigel plugs containing PlGF and heparin (positive control) or containing heparin only (negative control). The macroscopic appearance of typical plugs from each experimental group is shown. Histograms represent the mean value ($n = 6$) of the Hb content, expressed as OD/100 mg of matrigel plug. Bars: +SD values. Positive control of PARP-1 KO mice versus positive control of WT mice: $P = 0.004$ (∗) (Student's *t*-test).

neovascular endothelial cells associated to the tumour mass.[22] The observed anti-angiogenic effect of PARP inhibitor derives from a decrease of endothelial cell migration in response to angiogenic factors. On the other hand, endothelial cell proliferation and adhesion to various components of the extracellular matrix is not affected by inhibition of PARP. Actually, during the preparation of this manuscript Rajesh and colleagues reported the *in vitro* inhibition of HUVEC migration by PARP inhibitors.[20,21] These authors also demonstrated that PARP inhibitors impaired VEGF- and FGF-induced proliferations, tube formation and prevented sprouting of rat aortic ring explants using an *ex vivo* assay.

Preclinical tumour models have shown that the therapeutic role of PARP inhibitor for cancer treatment and clinical trials is currently evaluating its efficacy for the treatment of advanced malignant melanoma in combination with temozolomide or as single agent for advanced tumours with loss of BRCA1/2 function.[11,12] In both cases, the strategy of using PARP inhibitor relies on its ability to hinder DNA repair, maximising DNA damage and triggering apoptosis. Another application of PARP inhibitors regards their use in combination with anticancer agents such as doxorubicin, cisplatin or irinotecan to reduce their untoward effects avoiding cell death due to PARP overactivation.[7,25–27] In the present study, we show that PARP inhibitor exerts an anti-angiogenic effect, which cannot be directly related to increased DNA damage, since it is observed at drug concentrations that do not affect the viability and the proliferative potential of endothelial cells.

A role of PARP-1 in angiogenesis has been recently implied by Martin-Oliva and colleagues.[19] The authors show that the inhibition of PARP activity delays tumour formation during skin carcinogenesis, which has been attributed to the modulation of transcription factors that are broadly involved in cell responses, including proliferation, inflammation and angiogenesis. Moreover, a reduction of tumour vasculature and downregulation of the expression of genes involved in angiogenesis was detected during carcinogenesis in the skin of mice treated with PARP inhibitor or of PARP-1 KO mice. Actually, fibroblasts exposed to 20 μM of the PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2*H*)-isoquinolinone or derived from PARP-1 KO mice showed a lack of induction of HIF-1α, a transcription factor involved in vessel formation, including that associated with tumour mass, primarily through upregulation of VEGF expression.[28] Indeed, our results indicate that PARP inhibition directly affects endothelial function, since exposure of endothelial cells to as low as 0.5–1 μM GPI 15427 abrogated their ability to migrate in response to VEGF or PlGF and to form tubular structures in response to matrigel, an artificial matrix that mimics the basement membrane. However, in this range of PARP inhibitor concentrations no modulation of HIF-1α induction by the hypoxia mimetic $CoCl_2$ was observed, perhaps due to different mechanisms between the indirect carcinogenesis-induced angiogenesis and the direct growth factor-induced one.

It has been recently demonstrated that the insulin-like growth factor-1, which is known to promote angiogenesis

through the upregulation of VEGF expression, inhibits PARP by phosphorylation and that this effect would contribute to increased VEGF transcription.[29] These data are consistent with our results that evidenced a new role of PARP in promoting the angiogenic response of endothelial cells to exogenous VEGF.

VEGF and PlGF, a member of the VEGF family, are both angiogenic factors involved in tumour neovascularisation.[30] VEGF regulates blood vessels by interacting with two cell surface tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1)/Flt-1 and VEGFR-2/KDR that is the primary receptor transmitting VEGF signals in endothelial cells. PlGF binds to VEGFR-1 as homodimer or to VEGFR-2 as a heterodimer with VEGF and it has been found to be overexpressed in a variety of human cancers.[31] Different signal transduction pathways are triggered by VEGFRs and those implicated in endothelial cell migration include focal adhesion kinase (FAK) and the extracellular signal-regulated kinase 1/2 cascade (ERK1/2).[32] The inhibitory effect exerted by GPI 15427 on endothelial cell migration is observed when cells are stimulated by VEGF or PlGF, but not in response to EGF, suggesting a potential influence of the PARP inhibitor on VEGFRs signalling. However, the inhibitory effect of the PARP inhibitor GPI 15427 cannot be attributed to modulation of the phosphorylation status of FAK and ERK1/2 in response to VEGF and PlGF (data not shown).

It cannot be excluded that the compromised response of endothelial cells to angiogenic stimuli detected after treatment with PARP inhibitor or in the *in vivo* model of PARP-1 gene deletion might be due to the impaired ability of PARP-1 to control the transcription of specific genes, which might affect the response to VEGF or PlGF. PARP-1 regulates transcription by at least two different mechanisms: modulating chromatin structure and the function of a number of transcription factors. This activity is accomplished by PARP-1 through a direct protein–protein interaction or through poly (ADP-ribosyl)ation of the target molecule.[33] Poly (ADP-ribosyl)ation of histones loosens chromatin and renders genes accessible to the transcriptional machinery. Moreover, noncovalent interaction of histones with poly(ADP-ribose), as free polymer or attached to proteins, may also allow the release of histones from nucleosome rendering DNA available for transcription.[33] Even though in cells not subjected to DNA damage the average length of ADP-ribose polymers is shorter than in cells exposed to genotoxic injury, ADP-ribose polymers may still participate to regulate several functions also in intact cells.

In conclusion, these findings provide a novel therapeutic implication to the use of PARP inhibitors for cancer treatment. In addition to DNA repair mechanisms, targeting PARP may also suppress angiogenesis, which is an essential requirement for the growth of primary tumours and for the metastatic process as well. Further studies are requested to elucidate the molecular mechanisms involved in the anti-angiogenic effect of PARP inhibition.

Conflict of interest statement

None declared.

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