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Down-Regulation of Flt-1 Gene Expression by the Proteasome Inhibitor MG262

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Abstract The mechanisms involved in the anti-angiogenic actions of the proteasome inhibitors are poorly understood. Here, we report that the gene expression of the VEGF receptor Flt-1 (vascular endothelial growth factor receptor 1) was down-regulated by the reversible proteasome inhibitor MG262 in explant cultures of the developing chicken pecten oculi, a vascular organ consisting of endothelial cells, pericytes, and macrophages. In addition, the inhibitor prevented the induction of Flt-1 by lipopolysaccharide (LPS) in macrophages and down-regulated the expression of Flt-1 after LPS induction. Flt-1 gene expression was also down regulated by MG262 in cultures of human microvascular endothelial cells. Interestingly, a transcript of Flt-1, coding for a soluble form of the receptor (sFlt-1) with anti-angiogenic properties, was not down-regulated in the same extent. Only a small decrease in the expression of VEGF and Ang-2 was detected in the pecten oculi upon inhibition of the proteasome, while no major changes were observed in the expression of other angiogenic molecules, such as KDR or Ang-1. Since recent experiments have demonstrated the importance of anti-Flt-1 therapy in the inhibition of tumor angiogenesis, retinal angiogenesis, arthritis, and atherosclerosis (Luttun et al. [2002]: Nat Med 8:831–840), our observation on down-regulation of Flt-1 in microvascular endothelial cells and macrophages by MG262 supports the postulated role of the proteasome inhibitors as potential candidates for therapeutic modulation of angiogenesis and inflammation. J. Cell. Biochem. 89: 1138–1147, 2003.

Key words: angiogenesis; Ang-1; Ang-2; microvascular endothelial cells; KDR; lipopolysaccharide; macrophages; pecten oculi; sFlt-1; VEGF; VEGFR-1; VEGFR-2

Reversible proteasome inhibitors have recently emerged as a promising approach to the treatment of multiple forms of cancer and inflammatory diseases (for recent reviews see Adams, 2002; Almond and Cohen, 2002; Garcia-Echeverria, 2002; Goldberg and Rock, 2002). The proteasome is a large ATP-dependent multisubunit protease involved in the rapid degradation of intracellular proteins that regulate transcription, cell cycle, apoptosis, cell adhesion, antigen presentation by MHC class I molecules, and angiogenesis. The proteasome is also essential for the rapid elimination of abnormal proteins [Ciechanover, 1994; Hershko, 1997; Baumeister et al., 1998; Brooks et al., 2000; Doherty et al., 2002]. From the pivotal role of the proteasome in cellular physiology, we would expect indiscriminate changes in cell function as a consequence of the action of proteasome inhibitors. However, it has been demonstrated that only a limited number of genes change in expression in *S. cerevisae* after proteasome inhibition [Fleming et al., 2002]. Also in human tumor cells, a coordinated pattern of transcriptional events, consisting of down-regulation of transcripts involved in key growth/survival signaling pathways, and up-regulation of molecules implicated in pro-apoptotic pathways, has been described as the effect of the reversible proteasome inhibitor PS-341 [Mitsiades et al., 2002].

The main question to be examined in this paper is whether or not the reported anti-angiogenic action of the proteasome inhibitors [Oikawa et al., 1998; Sunwoo et al., 2001;

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LeBlanc et al., 2002] is the consequence of a limited selective action on the expression of certain angiogenic and anti-angiogenic molecules in cells involved in blood vessel formation. So far, only has been reported the down-regulation by the proteasome inhibitor PS-341 of few angiogenic molecules, such as VEGF and the angiogenic cytokine GRO-α [Sunwoo et al., 2001]. We have used a reversible proteasome inhibitor, the peptide boronate MG262, in a new model of angiogenesis, the developing chicken pecten oculi cultured in vitro. The pecten oculi, which consists of endothelial cells, periendothelial pigmented glial cells, and numerous macrophages, protrudes in the vitreous, and provides nutrition to the inner avian avascular retina [Navascues et al., 1995; Liebner et al., 1997]. This fascinating organ has been used previously as an in vivo model of blood–brain barrier [Gerhardt et al., 1996]. During the development of the chicken pecten oculi, we have determined the expression of vascular endothelial growth factor (VEGF), its main receptors, the angiopoietins (Ang-1, Ang-2), and other angiogenic and anti-angiogenic molecules (unpublished results).

It is well known that VEGF induces angiogenesis by interacting with two tyrosine kinase receptors: Flt-1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2) [Mustonen and Alitalo, 1995; Shibuya, 2001]. Flt-1 shows several unique properties compared with KDR [Waltenberger et al., 1994; Seetharam et al., 1995]: the extracellular domain of Flt-1 binds VEGF with an affinity tenfold higher than that of KDR; however, the tyrosine kinase activity of Flt-1 is one order of magnitude lower than that of KDR. The Flt-1 gene encodes two major transcripts, one coding for the full-length tyrosine kinase receptor, and the other coding for a soluble form of Flt-1, consisting of the N-terminal extracellular domain of the receptor and a short carboxy-terminal region derived from an intron [Kendall and Thomas, 1993]. The soluble form of Flt-1 (sFlt-1) binds VEGF with high affinity and suppresses its angiogenic and hyperpermeability actions [He et al., 1999]. In addition to VEGF and its receptors, the angiopoietins Ang-1 and Ang-2 are also involved in the control of angiogenesis. Ang-1 is an angiogenic factor that signals through the tyrosine receptor Tie-2 [Davis et al., 1996]. Ang-2, a natural antagonist of Ang-1, induces angiogenesis in the presence of VEGF and vascular regression in its absence [Maisonpierre et al., 1997; Mezquita et al., 1999].

We have used explant cultures of the whole chick embryo pecten oculi and cultures of microvascular endothelial cells and macrophages to explore the effect of the proteasome inhibitor MG262 on the coordinate expression of VEGF, Flt-1, KDR, and the angiopoietins Ang-1 and Ang-2. We report here that the proteasome inhibitor MG262 down-regulates the expression of Flt-1 in the pecten oculi, in microvascular endothelial cells, and also in macrophages that have been activated with lipopolysaccharide (LPS). Interestingly, the expression of one of the Flt-1 transcripts, coding for an anti-angiogenic soluble form of the receptor Flt-1 (sFlt-1), was not down-regulated significantly by the inhibitor. A small decrease was observed in the expression of other angiogenic molecules, such as VEGF and Ang-2, while no major changes occurred in the expression of KDR and Ang-1.

MATERIALS AND METHODS

Whole Pecten Oculi Explant Cultures

Fertilized eggs from White Leghorn chickens (*Gallus domesticus*) were incubated at 60% humidity and 37.8°C in a forced-draft turning incubator (Masalles G-180). Embryos on day 15 of incubation were sacrificed by decapitation. Eyes were immediately removed and placed in PBS. An incision was made slightly posterior to the corneal limbus and the anterior sections and lens were discarded. Then, the vitreous humor containing the pecten oculi was gently removed. The pecten oculi, clearly identified within the vitreous by its black color and morphology, was suspended in culture medium surrounded by a small amount of vitreous humor. Explants were maintained in EBM-2 medium (Clonetics, Cambrex, Verviers, Belgium) supplemented with 10% chicken serum and 0.05 mg/ml of gentamicin for 16 h in a 5% CO_2 atmosphere at 37°C in the absence or in the presence of the proteasome inhibitor MG262 (Calbiochem, Bad Soden, Germany) to a final concentration of 1 μM.

Cell Cultures

The HD11 chicken macrophages, kindly provided by P. Jurdic (École Normale Supérieure, Lyon, France), were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) supplemented with 5% chicken serum, 5% fetal calf serum, 10% tryptose phosphate broth,

and 1% penicillin–streptomycin. Human microvascular dermal neonatal endothelial cells (HMVEC-d-Neo) were obtained from Clonetics (cc-2516) and cultured following the provider's recommendations and media. Cells were maintained in a 5% CO_2 atmosphere at 37°C. HD11 macrophages were stimulated with 100 ng/ml LPS for 12 h. The proteasome inhibitor MG262 was added to subconfluent cells to a final concentration of 500 nM for 12 h.

Preparation of RNA, Electrophoresis, Northern Hybridization, and Immunological Detection

Explant cultures of the pecten oculi, corresponding to 20–60 organs, were sedimented at 2,000*g* for 10 min, and extracted with Trizol (Invitrogen) according to the specifications of the manufacturer. The procedures used for preparation of RNA, electrophoresis, Northern blot analysis, and immunological detection have been previously described [Mezquita et al., 1999].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First-strand cDNA was obtained using Moloney Murine Leukemia virus (M-MuLV) reverse transcriptase, random hexanucleotides, and 1 μg of total RNA in a final volume of 50 μl (Ready-To-Go RT-PCR Beads, Amersham Biosciences, Little Chalfont, UK) as recommended by the manufacturer. After heat-inactivation of the reverse transcriptase, PCR amplification was started by adding 10 picomol of each specific primer (two-step protocol). PCR conditions were as follows: a denaturing step of 5 min at 94°C, 23–32 cycles of 1 min at 95°C, 1 min at 3–5°C over the annealing temperature of the primers and 3 min at 72°C, then a final extension step of 7 min at 72°C. The number of cycles was adjusted with preliminary experiments to avoid saturation of the PCR product. One-tenth of each PCR product was analyzed by electrophoresis in a 1.5% agarose gel as described [Mezquita et al., 1999].

Primers and Probes

The sequences of primers and probes are shown in Table I. The position of the primers expanded at least one intron to characterize PCR products after RNA splicing and to discriminate from genomic contamination. In addition, when possible, the selected primers allowed detection of different isoforms resulting from alternative splicing. Rapid amplification of 3′-end (RACE-3′) of sFlt-1 was done with an upstream specific primer, located close to the end of the coding sequence (Table I, sequence #11) and two specific nested primers, N1 and N2 (Table I, sequences #12 and #13). The downstream primers were NotI-d$(T)_{18}$ from Amersham Pharmacia Biotech (Table I, sequence #14), and the N primer (Table I, sequence #15). To detect both full-length and soluble chicken Flt-1 by Northern blot analysis, probes were made using the Flt-1 forward and reverse primers (Table I, sequences #9 and #10), which amplify the tyrosine kinase domain (Fig. 4; 1) and the chicken sFlt-1 primers forward C and reverse S (Table I, sequences #16 and #17), located at the carboxi-terminal domain of sFlt-1 (Fig. 4; 2). A 5′-digoxigenin labeled oligonucleotide (Thermo Hybaid, Ulm, Germany) from the specific sFlt-1 last exon (Table I, sequence #18) was used for specific detection of sFlt-1 mRNAs (Fig. 4; 3). Probes for VEGF, KDR, Ang-1, and Ang-2 detection were prepared from the corresponding forward and reverse primers (Table I). Human Flt-1 kinase domain primers were used to obtain a probe that hybridized with the full-length Flt-1 mRNA (Table I, primers #25 and #26). Human Flt-1 N-terminal primers were used to obtain a probe that hybridized to both full-length and soluble Flt-1 forms (Table I, primers #27 and #28).

Purification of PCR Products, Cloning, and Sequencing

PCR products were purified free of oligonucleotides for direct sequencing or cloned and sequenced in a Licor MWG-Biotech GmbH automatic sequencer as previously described [Mezquita et al., 1999]. The sequencing primers, labeled with IRD800 (Thermo Hybaid), were the forward and reverse primers from the vector (Table I, sequences #19 and #20) and the specific primers (Table I, sequences #21 and #22).

RESULTS

Flt-1 Gene Expression Is Down-Regulated in Explants of Whole Chick Embryo Pecten Oculi Incubated in the Presence of the Proteasome Inhibitor MG262

Explant cultures of the whole pecten oculi of 15-day-old chick embryos were established in an attempt to preserve the in situ tissue

TABLE I. Primers and Probes Used for RT-PCR, Northern Blot Analysis, RACE-3′, and DNA Sequencing

cDNA	Type	Sequence	Number	Position	Accession no.
VEGF	Forward	CAACCATGAACTTTCTGCTCAC	1	1–22	ABO11078
Chicken	Reverse	TTTCCGCTGCTCACCGTCTC	2	647–666	
Ang-2	Forward	GATACAACGCCTTCGGGAAG	3	297–316	
Chicken	Reverse	GCTTGTCTTCCATCTCAAGAACTC	4	29,120–29,143	AJ289777
Ang-1	Forward	AGATGTATACCAATCTGGTTTTAAC	5	1–25	AJ539549
Chicken	Reverse	CAGCACCATGCAGGATCAGG	6	391–410	
KDR	Forward	GCCAGCAAGTGGGAGTTTCC	7	2,488–2,507	X83288
Quail	Reverse	CTCCGTAGATATGCCGAGAGATTTC	8	2,783–2,807	
Flt-1	Forward K	CACAGCGAGTGAGTACAAAGC	9	2,843–2,863	ABO65372
Chicken	Reverse K	TCCAGCATGATTTGGTAGATCTC	10	3,627–3,649	
sFlt-1	Forward	TTTCCCTGGCACACTCAGGC	11	2,113–2,132	ABO65373
Chicken	Forward N1	CAGAGGTGAGCACTGCAAC	12	2,201–2,219	
	Forward N2	GTATCAGGTGTCTCATATCATCTTG	13	2,316–2,340	
	Reverse Not I-d$(T)_{18}$	AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$	14		
	Reverse N	GAAGAATTCGCGGCCGCAGG	15		
	Forward C	CATAGGAAACAGGATCCAGAGC	16	1,745–1,766	ABO65373
	Reverse S	TGAGGATCCGAGAGTAAACAGC	17	2,226–2,247	
	Dig oligo probe	TACAATCATTCCTTGTGTTCTTATATTTGAG-GATCCGAGAGTAAACAGCC	18	2,225–2,274	ABO65373
	IRD800VF	AGGGTTTTCCCAGTCACGACGTT	19		
	IRD800VR	GAGCGGATAACAATTTCACACAGG	20		
	IRD800S1	CAATCAGAGGTGAGCACTGCAAC	21	2,197–2,219	ABO65373
	IRD800S2	TCAGGTGTCTCATATCATCTTG	22	2,319–2,340	
S17	Forward	TACACCCGTCTGGGCAACGAC	23	98–118	AY215074
Chicken	Reverse	CCGCTGGATGCGCTTCATCAG	24	206–226	
Flt-1	Forward K	GATGTTGAGGAAGAGGAGGATT	25	3,184–3,205	X51602
Human	Reverse K	AAGCTAGTTTCCTGGGGGTATA	26	4,308–4,329	
Flt-1	Forward N	GCTCACCATGGTCAGCTACTG	27	243–263	X51602
Human	Reverse N	CAGTGATGTTAGGTGACGTAACC	28	726–748	
β-Actin	Forward	CCTCGCCTTTGCCGATCC	29	27–44	BC016045
Human	Reverse	GGATCTTCATGAGGTAGTCAG	30	632–652	

RACE–3′, rapid amplification of 3′-end; RT-PCR, reverse transcriptase-polymerase chain reaction; VEGF, vascular endothelial growth factor.

architecture of this vascular organ. The explants were incubated for 16 h, as described in "Materials and Methods," in the absence or in the presence of the proteasome inhibitor MG262 to a final concentration of 1 μM. RT-PCR analysis demonstrated that the explant cultures of pecten expressed Flt-1, KDR, VEGF, Ang-1, and Ang-2 (Fig. 1). With chicken Flt-1 primers, a single band was obtained at the expected size of 807 bp. This band corresponded to full-length Flt-1 mRNA. The level of Flt-1 transcripts markedly decreased in the presence of the proteasome inhibitor. KDR primers yielded a band of 320 bp. The level of the KDR transcripts was not changed by the presence of the proteasome inhibitor. VEGF-A primers gave signals at the expected sizes of 462, 534, and 594 bp, which corresponded to VEGF-A_{122}, VEGF-A_{146}, and VEGF-A_{166} mRNAs, respectively [Sugishita et al., 2000]. The levels of these transcripts were not significantly changed in the pecten by exposure to the proteasome inhibitor. Ang-1 primers yielded the expected band of 411 bp. Ang-2 primers gave bands to the estimated 543 and 387 bp products that corresponded to the previously described isoforms of Ang-2, Ang-2A, and Ang-2C [Mezquita et al., 2000]. No major changes in the expression of Ang-1 and Ang-2 were detected by the action of the proteasome inhibitor.

In addition to RT-PCR, we have investigated by Northern blot analyses the effect of the proteasome inhibitor in the relative expression levels of Flt-1, VEGF, KDR, Ang-1, and Ang-2 in explants of the whole pecten. With cDNA probes that detect both the full-length Flt-1 and the soluble sFlt-1 transcripts three main bands were detected (Fig. 2; 1 and 2). Using a specific probe for the transcripts that codify the soluble forms of Flt-1, only the two bands of higher mobility were detected (Fig. 2; 3 and 4). Both the full-length Flt-1 transcript and the larger sFlt-1 transcript markedly decreased, tenfold and fourfold, respectively, upon exposure of the pecten to the inhibitor (Fig. 2; 2 and 4). However, the level of the smaller form of the sFlt-1 transcript did not decrease by the action of the MG262 (Fig. 2; 2 and 4). Only a small decrease in the expression of VEGF and Ang-2 was detected, while no major changes were

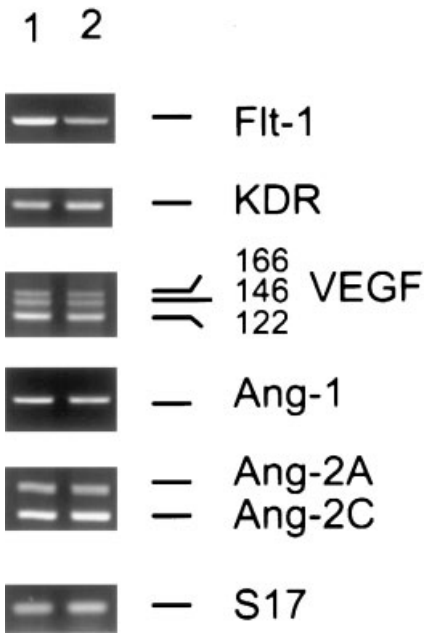


Fig. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Flt-1, KDR, VEGF, Ang-1, and Ang-2 expression in explant cultures of whole pecten oculi incubated in the absence (**1**) or in the presence (**2**) of the proteasome inhibitor MG262. After 16 h of incubation, total RNA was extracted from the pecten and analyzed by RT-PCR with specific primers as described under ''Materials and Methods.'' The mRNA transcript of chicken S17 ribosomal protein [Trueb et al., 1988] was used as a loading control. Results were similar in three independent experiments.

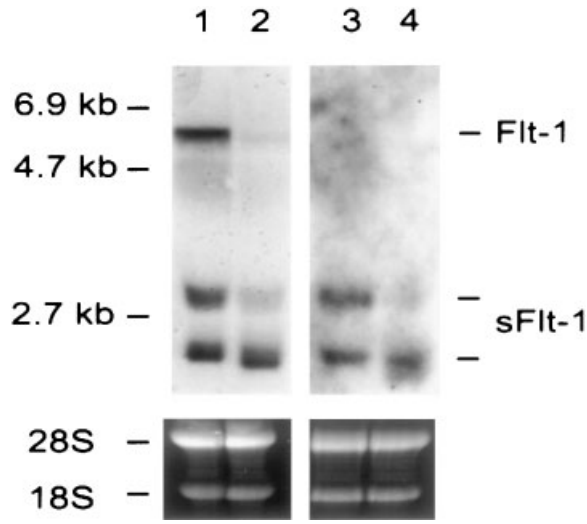


Fig. 2. Northern blot analysis of total RNA from pecten oculi incubated in the absence (**1** and **3**) or in the presence (**2** and **4**) of the proteasome inhibitor MG262. After 16 h of incubation, total RNA was extracted from the pecten and analyzed by Northern blot. A mixture of probes 1 and 2 (Fig. 4) detects both the full-length and the soluble sFlt-1 (lanes 1 and 2). Probe 3 (Fig. 4) detects specifically sFlt-1 transcripts (lanes 3 and 4). Panels with ribosomal RNAs show the equalization of RNA samples used for analysis. Results were similar in three independent experiments.

observed in other angiogenic molecules such as KDR and Ang-1 (Fig. 3).

Characterization of Two Forms of Chicken sFlt-1 that Differ in the Length of the 3′-UTR

As it is mentioned in the previous section, two bands corresponding to sFlt-1 transcripts were detected by Northern analysis. Previously, only one chicken sFlt-1 mRNA has been characterized [Yamaguchi et al., 2002]. We report here the sequences of two chicken sFlt-1 mRNAs that differ in the length of the 3′-UTR (Fig. 4). Sequencing products of 3′-RACE of chicken sFlt-1, we have identified two forms of sFlt-1, one with a short 3′-UTR and the other with a long 3′-UTR. The 3′-end of the short form, corresponding to exon 13 of the gene, includes 93 nt of coding sequence, an inframe stop codon, and 18 nt of non-coding sequence followed by a poly(A)+ tail. The polyadenylation signals ATTAAA is located 18 nt upstream the poly(A)+ tail. This signal is conserved in mouse and human sFlt-1 [He et al., 1999]. A second ATTAAA sequence, located 6 nt upstream the poly(A)+ tail is present in chicken and human sFlt-1 [He et al., 1999]. The nucleotide sequence of the 3′-end of the short form of chicken sFlt-1 showed 92 and 83% identities with the corresponding short forms of human and mouse sFlt-1, respectively. The 3′-UTR of the long form of sFlt-1 (696 nt) contained the cytoplasmic polyadenylation element (CPE) TTTTTTAT and the polyadenylation signal AATAAA located 16 nt upstream from the poly(A)+ tail. The 3′-UTR of the long sFlt-1 contained the mRNA destabilizing sequence TTATTTATT included in a segment of 28 nt highly conserved in the 3′-UTR of the mouse and human sFlt-1. The absence of destabilizing sequences in the short form of sFlt-1 could explain the relative abundance of this transcript before and after exposure to the proteasome inhibitor MG262.

Flt-1 Gene Expression Is Down-Regulated in Cultures of Human Microvascular Endothelial Cells Incubated in the Presence of the Proteasome Inhibitor MG262

HMVEC-d-Neo were incubated for 12 h in the absence or in the presence of the proteasome inhibitor MG262 to a final concentration of 500 nM. RT-PCR and Northern analyses showed a marked decrease in the expression of Flt-1

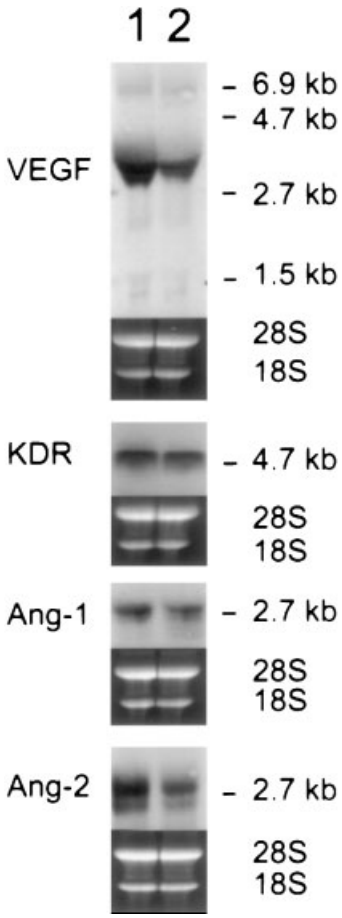


Fig. 3. Northern blot analysis of total RNA from pecten oculi incubated in the absence (**1**) or in the presence (**2**) of the proteasome inhibitor using specific probes for detecting VEGF, KDR, Ang-1, and Ang-2 transcripts. Panels with ribosomal RNAs show the equalization of RNA samples used for analysis. Results were similar in three independent experiments.

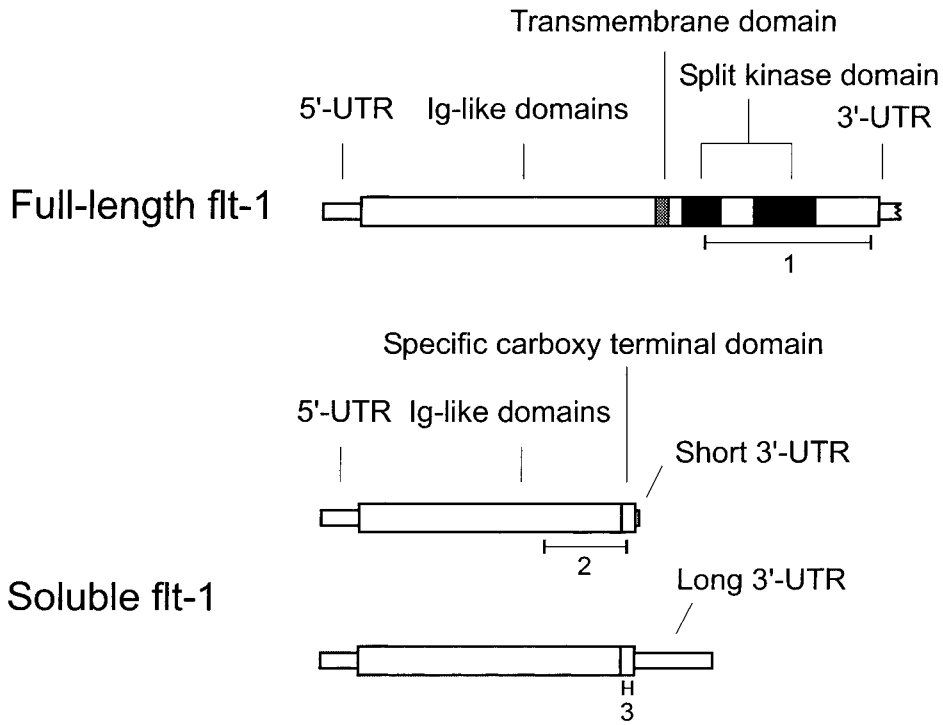
(Fig. 5). With human Flt-1 primers, from the tyrosine kinase domain, a single band was obtained at the expected 1,146 bp that corresponded to the full-length Flt-1 mRNA. The level of Flt-1 transcripts markedly decreased in the presence of the proteasome inhibitor. By Northern analysis, using a cDNA probe from the tyrosine kinase domain, two bands were obtained: a main band that corresponded to the full-length human Flt-1 transcripts and a minor band of higher mobility. The expression of the full-length Flt-1 transcript markedly decreased upon exposure of the microvascular endothelial cell culture to the proteasome inhibitor. Using a cDNA probe, from the N-terminal extracellular domain of Flt-1, three main bands were detected that decreased upon incubation with the proteasome inhibitor.

Proteasome Inhibitor MG262 Prevents the LPS Induction of Flt-1 Gene Expression in Chicken Macrophages and Down-Regulates the Expression After LPS Induction

Resting chicken macrophages expressed low levels of Flt-1 mRNA (Fig. 6; 1). Exposure of chicken macrophages to LPS led to a significant up-regulation of both full-length and soluble Flt-1 mRNAs (Fig. 6; 4). A similar observation was previously reported in human monocytes [Barleon et al., 1996]. This induction was completely prevented in the presence of the proteasome inhibitor MG262 (Fig. 6; 3). After induction of Flt-1 gene expression by LPS, the proteasome inhibitor MG262 down-regulated the expression of the gene, particularly the mRNA coding for the full-length receptor and the mRNA coding for the long form of sFlt-1 (Fig. 6; 6).

DISCUSSION

We have used the chicken pecten oculi, an ocular vascular organ consisting of endothelial cells, pericytes, and abundant macrophages, as a new research model to study the coordinated expression of several angiogenic molecules such as VEGF, KDR, Flt-1, Ang-1, and Ang-2 upon the exposure to the proteasome inhibitor MG262. Flt-1 gene expression was down-regulated in explants of whole chick embryo pecten oculi incubated in the presence of the proteasome inhibitor, while only small decreases of VEGF and Ang-2 and no major changes of KDR and Ang-1 occurred. Not all forms of Flt-1 were down-regulated in the same extent by the proteasome inhibitor. The level of the smaller form of sFlt-1 did not decrease after treatment with the inhibitor. We have determined the sequence of two major forms of sFLt-1 transcripts that differ in the length of their 3′-UTR. A higher stability of the shortest form of sFlt-1, due to the lack of destabilizing sequences in its 3′-UTR, could explain the relative abundance of this transcript after treatment with the proteasome inhibitor MG262. In addition to the pecten, we have used cell cultures of chicken macrophages and human microvascular endothelial cells to study the effect of the proteasome inhibitor MG262 on the expression of Flt-1. The inhibitor prevented the induction of Flt-1 by LPS in macrophages and down-regulated the expression of Flt-1 after LPS induction. Flt-1 gene expression was also down-regulated by



3'end of sFlt-1with a short 3'-UTR

GGTGAGCACTGCAACAAAAAGGCTGTTTACTCTCGGATCCTCAAATATAAG
AACACAAGGAATGATTGTACAACACAAAGCAATGTAAAACATTAAAGGACT
CATTAAACAGTAAAAAAAAAAAAAAAAAAAAA

3'end of sFlt-1with a long 3'-UTR

GGTGAGCACTGCAACAAAAAGGCTGTTTACTCTCGGATCCTCAAATATAAGA
ACACAAGGAATGATTGTACAACACAAAGCAATGTAAAACATTAAAGGACTCA
TTAAACAGTATCAGGTGTCTCATATCATCTTGATTTCTTATTGCTGTTGCCA
CCCTTCAGGCCCTGAGGTGGTGCACATTCCCCCATGGGTTGGACGTAGGAGG
AATAGCAAACAAAATCTTTGCGTGAGCTTCCAGTTGAGTTCAGAYGGGTATT
TTCTGCTTTGACAAAGCACGTGGAAGCTGGGGTTTGGAAGACCCCCTGCGCT
ACATTTTGCATATTGTTCTTTTTTCTCTTGCTGTCTCCTGCTGAGTGATCAG
GAAAACTTTGGATCAATCTTTTCTTCTACCTTGGTATCAGACTCGTCAAAAG
TCAAAGCTGAATAAACTGATTAACTTTAAAAACTGCTGTTTTCAATCAACAA
CGGACATTTAATAAGTTAACGTTAATGCGCAGAATGAGCAGAACTCAGTTTG
CGATTGACTGGACAATATATTTTTTTATTTTAATCTGATCTTGATGGACTTAT
AAATATTACCTGCTGAGCAAGTTCTAATGCTTATTTATTTGAAATTATTTTC
TACACTAAAGTACATTAATCTTAAAAAGTATCTTCTAAAGGAAGTATGCAGG
GAAGAAATAAAAATGTTGATATTATGCACAGCTCTATCATATCATTTATAAT
AATGATGATTTAAAAACTGACAGACATGAACTATGTTTCTCCAAATAAATCC
AGGTAGATTTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Fig. 4. Schematic representation of full-length and soluble chicken Flt-1 cDNAs and nucleotide sequences of the 3′-end of chicken sFlt-1 transcripts. The positions of the probes used for Northern analysis were indicated by the numbers 1–3. Probe (**1**) detects the full-length Flt-1; probe (**2**) detects full-length and soluble forms of Flt-1; probe (**3**) detects specifically the soluble forms of Flt-1. The polyadenylation signals are underlined. The cytoplasmic polyadenylation element (CPE) is double underlined. The mRNA destabilizing sequence is boxed. The nucleotide sequences have been deposited in the EMBL database under accession nos. AJ539547 and AJ539548.

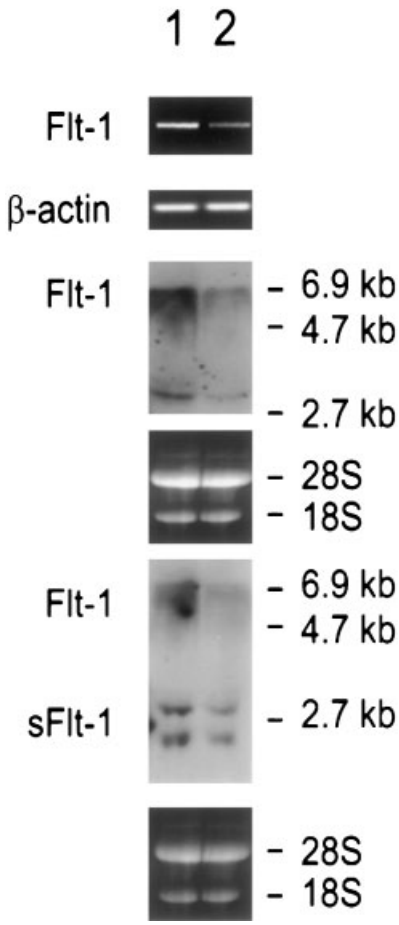


Fig. 5. RT-PCR and Northern analysis of Flt-1 expression in human microvascular endothelial cells incubated in the absence (**1**) or in the presence (**2**) of the proteasome inhibitor MG262. After 12 h of incubation, total RNA was extracted from the microvascular endothelial cells and analyzed by RT-PCR and Northern with specific primers and probes described under ''Materials and Methods.'' The mRNA transcript of β-actin was used as a loading control. Panels with ribosomal RNAs show the equalization of RNA samples used for analysis. Results were similar in three independent experiments.

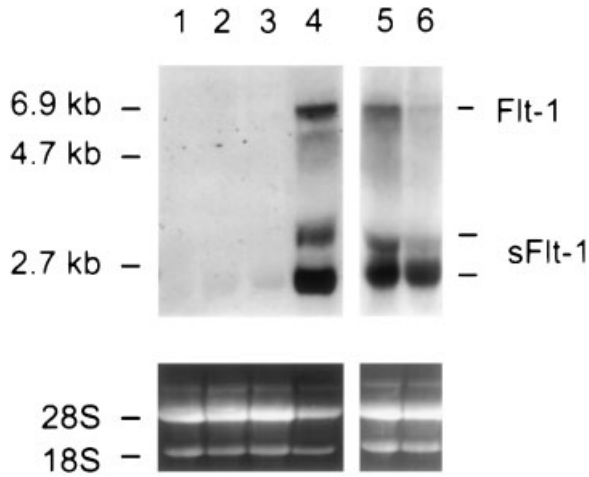


Fig. 6. Effect of lipopolysaccharide (LPS) on Flt-1 expression in chicken macrophages incubated in the absence (**1** and **4**) or in the presence (**2** and **3**) of the proteasome inhibitor MG262. Macrophages were incubated with 100 ng/ml LPS for 12 h (3,4) with or without the proteasome inhibitor. After LPS induction for 12 h, Flt-1 gene expression was down-regulated by the proteasome inhibitor MG262 (**6**). Two cDNA probes (1 and 2 in Fig. 4) were used for detecting both the full-length and the soluble sFlt-1 transcripts. Panels with ribosomal RNAs show the equalization of RNA samples used for analysis. Results were similar in three independent experiments.

MG262 in cultures of human microvascular endothelial cells.

Although the anti-angiogenic actions of the proteasome inhibitors have been well documented [Oikawa et al., 1998; Sunwoo et al., 2001; LeBlanc et al., 2002], only the down-regulation of few angiogenic molecules, such as VEGF and the cytokine GRO-α, has been reported [Sunwoo et al., 2001]. Our observation of down-regulation of Flt-1 expression in both macrophages and microvascular endothelial cells may have important implications. The increase in Flt-1 transcripts by LPS suggests an important role of the receptor in the function of monocytes/macrophages in vivo. It has been demonstrated that the migration of monocytes/macrophages in response to VEGF is mediated by Flt-1 [Barleon et al., 1996; Clauss et al., 1996; Lyden et al., 2001]. Activated Flt-1 myeloid cells release angiogenic factors such as VEGF, platelet derived growth factor (PDGF) and brain-neurotrophic factor (BDNF), which enhance vessel formation and stability [Donovan et al., 2000; Takakura et al., 2000]. One mechanism by which blocking Flt-1 expression with proteasome inhibitors may exert its anti-angiogenic effect is by interfering with the recruitment of macrophages.

Recently, it has been reported that an antibody against Flt-1 suppressed neovascularization in tumors and ischemic retina, and angiogenesis and inflammatory joint destruction in autoimmune arthritis [Luttun et al., 2002]. Anti-Flt-1 also reduced atherosclerotic plaque growth and vulnerability [Luttun et al., 2002]. In addition to the direct anti-angiogenic effects of blocking Flt-1 signaling, the anti-inflammatory effects of Flt-1-antibodies have been attributed to: (1) reduced mobilization of bone marrow-derived myeloid progenitors into the peripheral blood; (2) impaired infiltration of

Flt-1-expressing leukocytes in inflamed tissues; and (3) defective activation of myeloid cells [Luttun et al., 2002]. Flt-1 also mediates the development of lung metastasis inducing the expression of metalloproteinase MMP9 in lung endothelial cells and macrophages [Hiratsuka et al., 2002]. Thus, down-regulation of Flt-1 expression could be useful for the prevention of lung metastasis.

We have shown that not all forms of Flt-1 are down-regulated in the same extent by the proteasome inhibitor. The level of the smaller form of sFlt-1 transcripts did not decrease significantly after treatment with the inhibitor. Interestingly, sFlt-1 has a negative regulatory function in angiogenesis, possibly because its strong VEGF-trapping activity [Hiratsuka et al., 2001]. We do not know yet if the relative conservation of the small sFlt-1 transcript is accompanied by preservation of the corresponding protein. If this is the case, the relative maintenance of the anti-angiogenic sFlt-1, in relation to the full-length angiogenic Flt-1 tyrosine kinase receptor, may contribute to the anti-angiogenic properties of the proteasome inhibitors. In conclusion, among the angiogenic molecules studied in this paper, only the Flt-1 gene expression was markedly down-regulated by the proteasome inhibitor MG262. Due to the relevance of Flt-1 signaling in tumor angiogenesis and metastasis, and in inflammatory angiogenesis, our observation of down-regulation of Flt-1 gene expression by the proteasome inhibitor MG262, suggests that the inhibition of the proteasome may contribute to an effective therapy for cancer and inflammatory diseases.

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