Interleukin 1 induces hypoxia-inducible factor 1 in human gingival and synovial fibroblasts

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Rheumatoid arthritis and periodontitis are inflammatory diseases modulated by proinflammatory cytokines [e.g. interleukin (IL)-1 and tumour necrosis factor α], which activate local fibroblasts to do the following: (1) proliferate, (2) induce gene expression and (3) produce destructive metalloproteinases. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor (composed of HIF-1 α and HIF-1 β /aryl hydrocarbon receptor nuclear transporter) that is modulated by hypoxia. HIF-1 binds to and induces several genes containing an HIF-1 consensus-binding site, including vascular endothelial growth factor and several glycolytic enzymes. Through differential screening of a

human synovial fibroblast cDNA library, we identified HIF-1 α as a clone up-regulated by IL-1. The mRNA for HIF-1 α subunit was increased 3–4-fold by Northern blot analysis after cells had been incubated for 3 h in the presence of IL-1. In addition, IL-1 increased the binding of the heterodimer HIF-1 to the HIF consensus sequence. These results suggest that HIF-1 might have a role in inflammation, possibly in attempting to re-establish homeostasis.

Key words: periodontitis, proinflammatory cytokines, rheumatoid arthritis.

INTRODUCTION

Inflammatory diseases such as rheumatoid arthritis (RA) and periodontitis are characterized by the chronic, progressive destruction of connective tissue (reviewed in [1–3]). In chronically inflamed RA synovium and adult periodontal lesions, numerous blood vessels can be found within the proliferating tissue to provide nutrients to the inner cells of the thickened lining [4,5].

Proinflammatory cytokines [e.g. interleukin 1(IL-1) and tumour necrosis factor (TNF)- α] have a major role in maintaining the inflammatory state [6,7]. One important effect of IL-1 is to stimulate fibroblasts to produce destructive metalloproteinases such as collagenase [matrix metalloproteinase (MMP)-1] and stromelysin (MMP-3), which degrade components of the extracellular matrix in RA and periodontitis. However, cytokine-induced gene expression is also likely to contribute to the proliferation and tissue destruction associated with inflammatory diseases.

To determine which other genes are up-regulated in the presence of IL-1, we have previously reported the preparation of a differentially screened human synovial fibroblast (HSF) cDNA library [8]. Here we present the characterization of one of the cDNA species isolated by this method as hypoxia-inducible factor (HIF)-1 α (GenBank[®] accession no. U22431). HIF-1 is a heterodimeric transcription factor that has been found to modulate the cellular response to hypoxia [9]. The HIF heterodimer consists of HIF-1 α and HIF-1 β . HIF-1 β is also known as aryl hydrocarbon receptor nuclear translocator ('ARNT') because it can dimerize with the aryl hydrocarbon receptor as well as with HIF-1 α . Both HIF-1 α and HIF-1 β contain helix–loop–helix and PAS ('Per, ARNT, Sim') domains [10]. Whereas HIF-1 β is constitutively expressed under normoxic conditions, HIF-1 α is rapidly degraded by the ubiquitin–proteasome system. Under

hypoxic conditions (experimentally, 1% O₂/5% CO₂/94% N₂, or in the presence of CoCl₂, which mimics hypoxic conditions [11,12]), HIF-1 α protein accumulates, the heterodimer translocates to the nucleus and binds to a family of genes containing a specific sequence motif; these genes include erythropoietin (EPO), vascular endothelial growth factor (VEGF), and several glycolytic enzymes [13].

The present study demonstrates that the steady-state levels of HIF-1 α mRNA are increased on the addition of IL-1, as well as by TNF- α or lipopolysaccharide (LPS), to fibroblast cultures under normoxic conditions. Further, we show that IL-1 induces the binding of HIF-1 to a DNA consensus sequence in both gingival and synovial fibroblasts.

EXPERIMENTAL

Cell culture

Human synovial tissue samples were obtained from patients undergoing joint replacement or reconstructive surgery. Human gingival tissue samples were obtained from patients undergoing periodontal surgery. HSF and human gingival fibroblast (HGF) cultures were established from individual patients by enzymic dispersion of the samples and maintained in Eagle's minimal essential medium as previously described [14,15]. Cultures in passage 5–8 were used for the extraction of RNA or nuclear protein. Cells were serum-deprived overnight with the addition of insulin/transferrin/sodium selenite medium supplement (Sigma Chemical Co.) and then stimulated with IL-1 β (100 ng/ml final concentration; Dupont-Merck Corp.) and/or CoCl₂ (50 μ M final concentration). In other experiments cells were stimulated with TNF- α (10 ng/ml final concentration;

Abbreviations used: ATF, activating transcription factor; β -2, β -2-microglobulin; CREB, cAMP-response-element-binding protein; CHX, cycloheximide; EMSA, electrophoretic mobility-shift assay; EPO, erythropoietin; HGF, human gingival fibroblast; HSF, human synovial fibroblast; HIF, hypoxia-inducible factor; IL-1, interleukin 1; LPS, lipopolysaccharide; RA, rheumatoid arthritis; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

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Sigma Chemical Co.), with LPS (5 or 100 $\mu\text{g/ml}$, from *Salmonella typhimurium*; Calbiochem Corp.) or with cycloheximide (CHX, 100 ng/ml final concentration; Sigma Chemical Co.).

Library preparation and differential screening

A cDNA library was constructed from mRNA (FastTrack Kit; Invitrogen Corp.) isolated from cultured HSFs, derived from three or four individual patients, treated with IL-1 for 2, 4 or 6 h. The cDNA was generated and ligated into a Lambda Zap II vector (Stratagene Corp.) as described previously [8]. Duplicate lifts of plated library phage were probed with radiolabelled cDNA reverse transcribed from mRNA from either control or IL-1-induced HSFs in the presence of [α - ^{32}P]dCTP (Amersham Life Sciences). Individual plaques that hybridized differently with IL-1-induced cDNA probes than with control cDNA probes, and also showed a difference when secondarily screened, were excised *in vivo* to generate subclones in the Bluescript SK(-) vector (Stratagene Corp.).

Northern blot analysis

Total RNA was isolated from HSFs or HGFs, incubated in the presence or absence of cytokine, by the acid guanidinium thiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi [16]. RNA was subjected to electrophoresis in agarose/formaldehyde gels and transferred to Hybond nylon membrane (Hybond-N, Amersham Life Sciences) by standard methods [17]. DNA probes were prepared from low-melting-point agarose (Gibco BRL Life Technologies) gel slices of restriction-enzyme-digested plasmids containing HIF-1 α (obtained from the cDNA library) or β_2 -microglobulin (β_2 ; a gift from K. J. Soprano, Philadelphia, PA, U.S.A.). Probes were labelled by random priming with the Prime-It II kit (Stratagene Corp.). After prehybridization [50% (v/v) formamide/0.5% SDS/5 $\times$ SSC (where 1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate)/5 $\times$ Denhardt's solution/0.5 mg/ml denatured salmon sperm DNA] and hybridization with the radioactive probe at 42 $^{\circ}\text{C}$, the Northern blots were washed at 50 $^{\circ}\text{C}$ in 0.2 $\times$ SSC/10% (w/v) SDS/20 mM phosphate (pH 7.0). The blots were exposed to BioMax film (Eastman Kodak Co.). Developed films were quantified by densitometric analysis and normalized to β_2 .

Electrophoretic mobility-shift assay (EMSA) in HSFs and HGFs

Nuclear extracts were isolated by the method of Shreiber et al. [18] as described by Beck et al. [19], then quantified in mini-Bradford assays (Pierce Chemical Co.). Synthetic oligonucleotides corresponding to the human EPO HIF-1-binding site located in the 3' flanking sequence, 5'-GGGCCCTACGTGCTGTCTCACACAGCCTG-3' [19] were annealed and treated with T4 polynucleotide kinase in the presence of [γ - ^{32}P]ATP. The HIF-1-binding site as defined by Wang and Semenza [20] is underlined; the core motif is in bold [13]. Binding reactions were performed at 30 $^{\circ}\text{C}$ and contained 12 mM Hepes, pH 7.4, 8 μg of protein, 70 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% (v/v) glycerol, 4 μg of poly(dI-dC) and probe (160 000 c.p.m./ μl) in a final volume of 20 μl . Reaction products were subjected to PAGE [5% (w/v) gel] in 25 mM Tris base/190 mM glycine/1 mM EDTA (pH 8.0); the gel was then dried and exposed to film. Supershift EMSA used antiserum against either HIF-1 α or HIF-1 β (a gift from G. L. Semenza to J. C.).

RESULTS

Identification of HIF-1 α by differential screening of an HSF cDNA library

A cDNA library derived from cultured HSFs was differentially screened with probes obtained from mRNA from either control or IL-1-induced HSFs, as described in the Experimental section. One clone from the cDNA library that had a stronger signal in the presence of IL-1-induced cDNA probes than control probes was purified and sequenced; the partial nucleotide sequence was compared with the GenBank[®] database [21]. The sequence was identical with that encoding human HIF-1 α (accession no. U22431, nucleotides 1685–3678 [10]). The identification of a clone by differential screening implies that its corresponding gene might be under the regulation of IL-1; this was confirmed by Northern blot analysis.

IL-1-induced HIF-1 α mRNA in gingival and synovial fibroblasts

Steady-state levels of HIF-1 α mRNA derived from HGFs or HSFs were up-regulated in the presence of IL-1, as determined by Northern blot analysis (Figure 1). HIF-1 α mRNA was barely visible in control cells; however, levels increased after the addition of IL-1. Both HSFs (Figure 1, upper panels) and HGFs (Figure

Figure 1 Northern blot analysis of gingival (upper panels) and synovial (lower panels) fibroblasts in the presence of IL-1

Total RNA was purified from pooled HGF or HSF cultures established from two or three patients. RNA (20 μg) was run on each lane of a formaldehyde/agarose gel and transferred to nylon; the Northern blot was probed with radiolabelled cDNA corresponding to the mRNA for β_2 and HIF-1 α . The HGF or HSF cultures were either untreated (C) or treated with IL-1 for 1, 3 or 6 h, as shown. One HSF culture was treated with IL-1 and CHX together for 3 h (labelled in the lower panels as CHX 3). The washed blot was detected by autoradiography; HIF-1 α results were normalized to β_2 . The fold IL-1 induction (mean $\pm$ S.D. for three separate experiments) is shown under each band.

Figure 2 Northern blot analysis of synovial fibroblasts incubated for 3 h in the presence of IL-1, TNF- α , IL-1 and TNF- α , or LPS

HSF cultures established from three individual patients were each incubated for 3 h under the indicated experimental conditions. Cultures were either untreated (C) or treated for 3 h with IL-1 (100 ng/ml), TNF- α (10 ng/ml), both IL-1 and TNF- α at the same concentrations, or LPS (5 or 100 μ g/ml). Total RNA was purified and pooled from these cultures; 20 μ g was run in each lane on a formaldehyde/agarose gel and transferred to nylon. The Northern blot was probed with radiolabelled cDNA corresponding to the mRNA for β -2 and HIF-1 α . The washed blot was detected by autoradiography; HIF-1 α results were normalized to β -2. The fold induction is shown under each band.

Figure 3 EMSA showing DNA binding of HSF and HGF nuclear extracts to an HIF-1 consensus binding site

Nuclear extracts (5 μ g) from HGF or HSF cultures that had been either untreated (C) or treated for 6 h with IL-1 (100 ng/ml) or CoCl₂ (50 μ M) were incubated with a double-stranded DNA probe that had been end-labelled with ³²P. P, probe alone. The arrow shows the location of the inducible band. For comparison, a nuclear extract of HeLa cells incubated with CoCl₂ for 12 h was also incubated with the probe. The faster-migrating complexes result from the binding of ATF-1 and CREB [23] and are present in HSF, HGF and HeLa cells. The mixture was subjected to non-denaturing PAGE [5% (w/v) gel], dried and exposed to film.

1, lower panels) had an approx. 4-fold induction after 6 h of incubation with IL-1 when normalized to a housekeeping gene. The addition of CHX to synovial cells in the presence of IL-1 at 3 h did not significantly alter the induction of HIF-1 α by IL-1 (Figure 1, upper panels). Because CHX is an inhibitor of protein synthesis, these results suggest that protein synthesis *de novo* was not required for the induction of HIF-1 α mRNA by IL-1. The size of the HIF-1 α mRNA band in both HSFs and HGFs was approx. 3500 bases, which is consistent with reported values [10].

Figure 4 EMSA showing the time course of DNA binding of HSF nuclear extracts to an HIF-1 consensus binding site

Nuclear extracts (5 μ g) from HSF cultures that had been either untreated (C) or treated for the indicated durations (1, 3 or 6 h) with IL-1 (100 ng/ml) or CoCl₂ (50 μ M) were incubated with a double-stranded DNA probe that had been end-labelled with ³²P. P, probe alone. The mixture was subjected to non-denaturing PAGE [5% (w/v) gel], dried and exposed to film. The arrow shows the location of the inducible band. The faster-migrating complexes result from the binding of ATF-1 and CREB [23].

TNF- α and LPS also induce HIF-1 α mRNA

To identify other regulators of HIF-1 α , a Northern blot analysis of HSFs was performed after the cells had been incubated for 3 h with IL-1, TNF- α , a combination of IL-1 and TNF- α , or two different concentrations of LPS (Figure 2). IL-1, TNF- α and LPS all induced steady-state mRNA levels of HIF-1 α above control levels in comparison with a housekeeping gene (β -2). The two proinflammatory cytokines (IL-1 and TNF- α) induced HIF-1 α to approximately the same extent; they did not seem to act in synergy. Because LPS stimulates cytokine synthesis and secretion from cells [22], the increased levels of HIF-1 α in response to LPS probably reflect the autocrine or paracrine effects of IL-1 and/or TNF- α .

IL-1-induced binding to the HIF consensus sequence

To examine the effects of IL-1 on HIF-1 DNA binding, EMSAs were performed on nuclear extracts isolated from HGFs and HSFs previously incubated in the presence or absence of IL-1, and, for comparison, from HeLa cells subjected to CoCl₂. As shown in Figure 3, in addition to the IL-1-induced HIF-1 complex, constitutive bands with faster mobilities appeared in all EMSAs in the absence or presence of stimulus, primarily as two faster-migrating complexes. Previous reports attributed these complexes to the DNA-binding proteins activating transcription factor (ATF)-1 and cAMP-response-element-binding protein (CREB), binding being due to possible similarities between the HIF-1 and cAMP-response element consensus sequences [23]. However, after 6 h of treatment with either IL-1 or CoCl₂, an additional complex appeared nearer to the top of the gel (Figure 3, arrow). This new complex migrated at the same rate as that

Figure 5 EMSA detection of complex binding to the HIF-1 consensus DNA probe in the presence of antibodies against HIF-1 α and HIF-1 β

Nuclear extracts (5 μ g) from HGF (upper panel) or HSF (lower panel), either alone or after treatment for 6 h with IL-1 (100 ng/ml) or CoCl₂ (50 μ M), were incubated with a double-stranded DNA probe that had been end-labelled with ³²P. The filled arrow shows the location of the inducible band. The indicated lanes also contained antiserum specific to either HIF-1 α or HIF-1 β . The open arrowhead shows the approximate location of the supershifted band. The mixture was subjected to non-denaturing PAGE [5% (w/v) gel], dried and exposed to film.

found in HeLa cells subjected to CoCl₂ for 12 h, conditions known to induce HIF-1 binding.

To compare the time course of binding due to IL-1 or CoCl₂, EMSAs were performed with nuclear extracts from synovial fibroblasts incubated with either IL-1 or CoCl₂ for 1, 3 or 6 h (Figure 4). The induced complex was observed at 3 h of incubation with IL-1; the band was darker after 6 h of incubation. In contrast, the complex induced by CoCl₂ appeared earlier, at 1 and 3 h of incubation, possibly reflecting the different modes of regulation of IL-1 and CoCl₂. The intensities of the faster-migrating complexes vary and seem to have little effect on the HIF-1–probe complex. The anomalously high ‘constitutive’ band at 3 h in the presence of IL-1 could reflect IL-1-induced binding of ATF-1/CREB via the cAMP pathway [24].

When cells were incubated with both IL-1 and CoCl₂, the induced complex appeared with about the same intensity as that with CoCl₂ alone in HGFs (Figure 3) or in HSFs (Figures 3 and 4). With both IL-1 and CoCl₂, the induced complex was also not significantly darker than in the presence of IL-1 alone, suggesting that the two inducers were not acting in synergy.

Both HIF-1 α and HIF-1 β are present in the inducible binding complex

To determine the identity of the protein binding to the HIF DNA consensus sequence in the presence of IL-1, EMSAs were performed in the absence and in the presence of antisera specific for HIF-1 α and HIF-1 β (Figure 5). In extracts from both HSFs and HGFs, the IL-1-induced complex was supershifted by each of the two antisera, suggesting that this induced complex contained both HIF-1 α and HIF-1 β . In all lanes in which either anti-(HIF-1 α) or anti-(HIF-1 β) was present, there was a clear loss of the induced band (Figure 5, solid arrows). In some lanes, the ‘supershifted’ band seems weak in comparison with the original HIF-1 complexed band. The relative intensity of the HIF-1 complexed band in the absence or presence of antibody is a clearer indication of the outcome than the absence or presence of a supershifted band, because the degree of supershifted material depends on specific binding interactions of the antibodies with the DNA–protein complex.

DISCUSSION

It is well established that IL-1 is a major cytokine involved in the inflammatory process in both RA and periodontitis [6,7]. IL-1 acts directly on local fibroblasts in each condition, inducing a variety of genes and helping to create an ‘activated’ phenotype characterized by hyperplasia and invasiveness [4]. In RA, for example, the synovial lining, which is normally one or two cell layers thick, is converted into a ‘transformed-like’ tissue of up to 20 cells thick [25], owing to local proliferation or reduced apoptosis, or both [4]. Although this hyperplasia is accompanied by increased vascularization, the rheumatoid synovium undergoes multiple hypoxic events owing to increased fluid, causing raised pressure in the synovial cavity, compressing the blood vessels. These hypoxic events, followed by reperfusion and the generation of reactive oxygen species, result in repetitive trauma to the joint, which can be detrimental over time [26].

In an attempt to improve our understanding of the role of IL-1 in the ‘activation’ of fibroblasts during chronic inflammatory conditions, differential screening was performed to identify specific genes that are regulated by IL-1. With this approach it was determined that HIF-1 α mRNA is induced by IL-1 in human synovial and gingival fibroblasts under normoxic conditions. The induction of HIF-1 α might be a more general

inflammatory effect, because the mRNA is also induced by another proinflammatory cytokine, TNF- α . The mechanisms by which these proinflammatory cytokines regulate HIF-1 α mRNA levels are not clear; however, protein synthesis *de novo* does not seem to be required because the addition of CHX had no appreciable effect on steady-state levels of HIF-1 α mRNA. This finding is in contrast with that of Hellwig-Bürgel et al. [27], who reported no changes in HIF-1 α mRNA levels when HepG2 cultures were treated with TNF- α or IL-1 β under either normoxic or hypoxic conditions; the difference in these two studies is most probably due to cell-specific differences. Although there has been one report that HIF-1 α mRNA is induced by hypoxic conditions in hepatic cell lines [10], others have found the opposite [28,29]. It is now generally accepted that the hypoxic regulation of HIF-1 α occurs at the post-translational level by protein stabilization, followed upon reoxygenation by rapid degradation [30] through the ubiquitin-proteasome system [31].

It is interesting to note that IL-1-induced binding of the HIF-1 α protein occurs under normoxic conditions, an occurrence that also takes place in HepG2 cells [27]. Further, whereas the induction of HIF-1 protein DNA-binding activity by CoCl₂ is relatively quick and transient, the effect of IL-1 on HIF-1 binding activity is not fully apparent until 6 h after stimulation. These results suggest that different activation pathways are used and that the role of HIF-1 in response to hypoxia might be different from its role in response to inflammation. Although HIF-1 protein has been shown to bind and activate the transcription of a number of genes in several different cell types, its role during the inflammatory process has not previously been investigated.

Wenger et al. [32] divided genes affected by the HIF-1 protein activity into three classes: those that represent an adaptation of whole organisms to hypoxia, such as EPO, which stimulates the production of red blood cells; those that are local-acting factors, such as VEGF, which stimulates the production of new blood vessels; and those that have intracellular functions, such as glycolytic enzymes. Of the genes known to be induced by HIF-1, only the VEGF mRNA and protein have been shown to be induced by IL-1 in synovial fibroblasts [33–35]. In addition, RA patients have elevated serum concentrations of VEGF protein compared with normal subjects [35]. In periodontal patients, VEGF protein has been found in higher amounts in diseased sites than in clinically healthy sites [36]. Although, as stated earlier, new blood vessel formation is not sufficient to relieve hypoxia completely under inflammatory conditions, these results taken together do suggest a role for VEGF, and indirectly HIF-1, in inflammation.

The effect of IL-1 on EPO expression seems to be more complex. It is well established that hypoxic conditions result in up-regulation of the EPO gene but inflammatory cytokines blunt that response [37]. This is consistent with observations of anaemia in some inflammatory states [38], although the concentrations of circulating EPO protein in RA are elevated somewhat above normal [39]. Although the individual glycolytic enzymes have not been specifically shown to be up-regulated by IL-1, the activity of the glycolytic pathway in general is markedly increased in rheumatoid compared with normal synovium [40]. Whether these observations in RA patients are due to the presence of IL-1 or to local hypoxic conditions, or both, has not been elucidated. Inducing glycolysis is clearly beneficial in the short term by allowing the cells to generate energy under hypoxic conditions. However, the long-term consequences might be less favorable because the buildup of lactate from anaerobic glycolysis might actually cause damage by stimulating the additional release of inflammatory cytokines [41].

Recent reports of interaction between HIF-1 and p53 lend further support to the idea of HIF-1 as a stress response or 'survival' factor. Interaction with HIF-1 α seems to be necessary for stabilization of the p53 protein, allowing it to accumulate to levels necessary for functions related to cell cycle control and response to stress [42]. However, when levels of p53 are very high, such as when apoptosis is imminent, HIF-1 transactivation activity is inhibited [43], possibly via Mdm2-mediated ubiquitination and proteosomal degradation [44].

The functions attributed to HIF-1 via the gene activation of currently known genes suggest that HIF-1 is more likely to be involved in the attempted re-establishment of homeostasis than in contributing to the progression of tissue damage associated with chronic inflammation. Further research is needed to identify other genes activated by HIF-1 and to determine their roles in inflammation.

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REFERENCES

- Cunneane, G., Hummel, K. M., Muller-Ladner, U., Gay, R. E. and Gay, S. (1998) Mechanism of joint destruction in rheumatoid arthritis. *Arch. Immunol. Ther. Exp.* **46**, 1–7.
- Muller-Ladner, U., Gay, R. E. and Gay, S. (1997) Cellular pathways of joint destruction. *Curr. Opin. Rheumatol.* **9**, 213–220.
- Birkedal-Hansen, H. (1993) Role of matrix metalloproteinases in human periodontal diseases. *J. Periodontol.* **64**, 474–484.
- Walsh, D. A., Wade, M., Mapp, P. I. and Blake, D. R. (1998) Focally regulated endothelial proliferation and cell death in human synovium. *Am. J. Pathol.* **152**, 691–702.
- Bonakdar, M. P., Barber, P. M. and Newman, H. N. (1997) The vasculature in chronic adult periodontitis: a qualitative and quantitative study. *J. Periodontol.* **68**, 50–58.
- Badolato, R. and Oppenheim, J. J. (1996) Role of cytokines, acute-phase proteins, and chemokines in the progression of rheumatoid arthritis. *Semin. Arthritis Rheum.* **26**, 526–538.
- Tatakis, D. N. (1993) Interleukin-1 and bone metabolism: a review. *J. Periodontol.* **64**, 416–431.
- Fowler, Jr, M. J., Neff, M. S., Borghaei, R. C., Pease, E. A., Mochan, E. and Thornton, R. D. (1998) Induction of bone morphogenetic protein-2 by interleukin-1 in human fibroblasts. *Biochem. Biophys. Res. Commun.* **248**, 450–453.
- Wang, G. L. and Semenza, G. L. (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4304–4308.
- Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5510–5514.
- Goldberg, M. A., Dunning, S. P. and Bunn, H. F. (1988) Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* **242**, 1412–1415.
- Goldwasser, E., Jacobson, L. O., Fried, W. and Plzak, L. F. (1958) Studies on erythropoiesis. V. The effect of cobalt on the production of erythropoietin. *Blood* **13**, 55–60.
- Wenger, R. H. and Gassmann, M. (1997) Oxygen(es) and the hypoxia-inducible factor-1. *Biol. Chem.* **378**, 609–616.
- Mochan, E., Uhl, J. and Newton, R. (1986) Evidence that interleukin-1 induction of synovial cell plasminogen activator is mediated via prostaglandin E₂ and cAMP. *Arthritis Rheum.* **29**, 1078–1084.
- Docherty, A. J. and Murphy, G. (1990) The tissue metalloproteinase family and the inhibitor TIMP: a study using cDNAs and recombinant proteins. *Ann. Rheum. Dis.* **49**, 469–479.
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17**, 6419.

- 19 Beck, I., Weinmann, R. and Caro, J. (1993) Characterization of hypoxia-responsive enhancer in the human erythropoietin gene shows presence of hypoxia-inducible 120-Kd nuclear DNA-binding protein in erythropoietin-producing and nonproducing cells. *Blood* **82**, 704–711
- 20 Wang, G. L. and Semenza, G. L. (1993) Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J. Biol. Chem.* **268**, 21513–21518
- 21 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410
- 22 Agarwal, S., Baran, C., Plesco, N. P., Quintero, J. C., Langkamp, H. H., Johns, L. P. and Chandra, C. S. (1995) Synthesis of proinflammatory cytokines by human gingival fibroblasts in response to lipopolysaccharides and interleukin-1 β . *J. Periodontal Res.* **30**, 382–389
- 23 Kvietikova, I., Wenger, R. H., Marti, H. H. and Gassmann, M. (1995) The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. *Nucleic Acids Res.* **23**, 4542–4550
- 24 Chandra, G., Cogswell, J. P., Miller, L. R., Godlevski, M. M., Stinnett, S. W., Noel, S. L., Kadwell, S. H., Kost, T. A. and Gray, J. G. (1995) Cyclic AMP signaling pathways are important in IL-1 β transcriptional regulation. *J. Immunol.* **155**, 4535–4543
- 25 Firestein, G. S. (1998) Rheumatoid synovitis and pannus. in *Rheumatology* (Klippel, J. and Dieppe, P., eds), 2nd edn., sect. 5, pp. 13.1–24. Mosby, Philadelphia
- 26 Fairburn, K., Stevens, C. R., Winyard, P. G., Kus, M., Ward, R. J., Cunningham, J., Zaidi, M. and Blake, D. R. (1993) Oxidative stress and its control: a pathogenetic role in inflammatory joint disease. *Biochem. Soc. Trans.* **21**, 371–375
- 27 Hellwig-Burgel, T., Rutkowski, K., Metzen, E., Fandrey, J. and Jelkmann, W. (1999) Interleukin-1 β and tumor necrosis factor- α stimulate DNA binding of hypoxia-inducible factor-1. *Blood* **94**, 1561–1567
- 28 Gradin, K., McGuire, J., Wenger, R. H., Kvietikova, I., Whitelaw, M. L., Toftgard, R., Tora, L., Gassmann, M. and Poellinger, L. (1996) Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. *Mol. Cell. Biol.* **16**, 5221–5231
- 29 Wenger, R. H., Kvietikova, I., Rolfs, A., Gassmann, M. and Marti, H. H. (1997) Hypoxia-inducible factor-1 α is regulated at the post-mRNA level. *Kidney Int.* **51**, 560–563
- 30 Huang, L. E., Arany, Z., Livingston, D. M. and Bunn, H. F. (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. *J. Biol. Chem.* **271**, 32253–32259
- 31 Salceda, S. and Caro, J. (1997) Hypoxia-inducible factor 1 α (HIF-1 α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J. Biol. Chem.* **272**, 22642–22647
- 32 Wenger, R. H., Rolfs, A., Marti, H. H., Bauer, C. and Gassmann, M. (1995) Hypoxia, a novel inducer of acute phase gene expression in a human hepatoma cell line. *J. Biol. Chem.* **270**, 27865–27870
- 33 Ben-Av, P., Crofford, L. J., Wilder, R. L. and Hla, T. (1995) Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett.* **372**, 83–87
- 34 Jackson, J. R., Minton, J. A., Ho, M. L., Wei, N. and Winkler, J. D. (1997) Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1 β . *J. Rheumatol.* **24**, 1253–1259
- 35 Paleolog, E. M., Young, S., Stark, A. C., McCloskey, R. V., Feldmann, M. and Maini, R. N. (1998) Modulation of angiogenic vascular endothelial growth factor by tumor necrosis factor α and interleukin-1 in rheumatoid arthritis. *Arthritis Rheum.* **41**, 1258–1265
- 36 Booth, V., Young, S., Cruchley, A., Taichman, N. S. and Paleolog, E. (1998) Vascular endothelial growth factor in human periodontal disease. *J. Periodontal Res.* **33**, 491–499
- 37 Faquin, W. C., Schneider, T. J. and Goldberg, M. A. (1992) Effect of inflammatory cytokines on hypoxia-induced erythropoietin production. *Blood* **79**, 1987–1994
- 38 Jelkmann, W. E., Fandrey, J., Frede, S. and Pagel, H. (1994) Inhibition of erythropoietin production by cytokines. Implications for the anemia involved in inflammatory states. *Ann. N. Y. Acad. Sci.* **718**, 300–309; discussion, 309–311
- 39 Noe, G., Augustin, J., Hausdorf, S., Rich, I. N. and Kubanek, B. (1995) Serum erythropoietin and transferrin receptor levels in patients with rheumatoid arthritis. *Clin. Exp. Rheumatol.* **13**, 445–451
- 40 Mapp, P. I., Grootveld, M. C. and Blake, D. R. (1995) Hypoxia, oxidative stress and rheumatoid arthritis. *Br. Med. Bull.* **51**, 419–436
- 41 Niederman, R., Zhang, J. and Kashket, S. (1997) Short-chain carboxylic-acid-stimulated, PMN-mediated gingival inflammation. *Crit. Rev. Oral Biol. Med.* **8**, 269–290
- 42 An, W. G., Kanekal, M., Simon, M. C., Maltepe, E., Blagosklonny, M. V. and Neckers, L. M. (1998) Stabilization of wild-type p53 by hypoxia-inducible factor 1 α . *Nature (London)* **392**, 405–408
- 43 Blagosklonny, M. V., An, W. G., Romanova, L. Y., Trepel, J., Fojo, T. and Neckers, L. (1998) p53 inhibits hypoxia-inducible factor-stimulated transcription. *J. Biol. Chem.* **273**, 11995–11998
- 44 Ravi, R., Mookerjee, B., Bhujwala, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., Dillehay, L. E., Madan, A., Semenza, G. L. and Bedi, A. (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1 α . *Genes Dev.* **14**, 34–44

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