Relation of Vascular Endothelial Growth Factor Production to Expression and Regulation of Hypoxia-inducible Factor-1α and Hypoxia-inducible Factor-2α in Human Bladder Tumors and Cell Lines

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ABSTRACT

Hypoxia is an important regulator of vascular endothelial growth factor (VEGF) expression, and VEGF is associated with poor prognosis in bladder cancer. To investigate further the mechanisms of VEGF regulation, we examined VEGF expression by mRNA and protein analysis in four human bladder cancer cell lines, showing a progression from well to poorly differentiated phenotypes under varying conditions of confluence and hypoxia (0.1% O2) and with chemical mimics of hypoxia. Hypoxia significantly increased VEGF protein expression in all cell lines, although this effect was dependent on the degree of confluence. The superficial bladder cancer cell line RT4 lost hypoxia inducibility at confluence, whereas inducibility was maintained in the invasive cell lines 253J and EJ28. This pattern of VEGF expression in the invasive cell lines correlated with the expression of the transcription factor hypoxia inducible factor-1α (HIF-1α) and with hypoxia-inducible factor-2α (HIF-2α) and in RT4 correlated with a marked reduction in HIF-1α inducibility at confluence. Using the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor LY 294002, we show that this VEGF hypoxia-inducible pathway regulated by HIF-1α is distinct from a PI 3-kinase-dependent pathway, which regulates basal amounts of VEGF, but does not affect inducibility. Both HIF-1α and HIF-2α protein and mRNA were up-regulated in primary human bladder tumors (n = 12) compared with normal bladder specimens (n = 4), with significant intertumor variation. These results suggest that components of the hypoxia response pathway, including HIF-1α and HIF-2α, are important cofactors in the regulation of VEGF in bladder cancer and are therapeutic targets in this disease.

INTRODUCTION

Angiogenesis is essential to meet the metabolic requirements for tumor growth (1). One of the key angiogenic stimulators is VEGF. Not only is VEGF expression elevated and of prognostic significance in bladder, breast, gastric, and other malignancies (2), but key transfection and inhibitory experiments have confirmed its critical importance in angiogenesis and tumor growth (3–5).

Previously, we have demonstrated that VEGF is prognostic in superficial bladder cancer where high mRNA levels predict early stage progression and poor relapse-free survival (6). Elevated urinary VEGF protein levels provide similar prognostic and diagnostic information (7), with high levels being associated with high relapse rates and stage progression.

Hypoxia up-regulates VEGF expression (8), partly through increasing mRNA transcription and partly through increasing mRNA stability mediated through the RNA-binding protein HuR (9). Within tumors, VEGF mRNA colocalizes with hypoxic regions (10). The VEGF gene and several other genes regulated by hypoxia and involved in oxygen homeostasis are under the control of the transcription factor HIF-1 (11). HIF-1 comprises two subunits from the basic helix-loop-helix PAS domain protein family, HIF-1α and HIF-1β/ARNT (12). mRNA levels of HIF-1α are equivalent in normoxia and hypoxia (13), but in hypoxia there is inhibition of the O2-dependent degradation of HIF-1α protein, via the ubiquitin-proteasome pathway (14), recently shown to be regulated by the von Hippel-Lindau tumor suppressor gene product (15). There is an exponential increase in HIF-1α protein concentration, with decreasing oxygen tension, with a half-maximum response between 1.5 and 2.0% O2 and a maximum response at 0.5% O2 (16).

Because the HIF-1β/ARNT subunit is constitutively expressed and involved in other cellular processes, the HIF-1α subunit is thought to be the specific regulator of the hypoxia response (17). Loss of HIF-1α in embryonic stem cells signifi-
ificantly reduced solid-tumor growth, correlating with decreased expression of VEGF during hypoxia (18). We and others have demonstrated reduced in vivo tumor growth and angiogenesis with functional impairment of the HIF-1α/ARNT pathway, suggesting that the HIF-1α pathway is an important component of oncogenic angiogenesis (19, 20).

Another member of the helix-loop-helix family is endothelial PAS protein-1 (21–23). Endothelial PAS protein-1 shows 48% homology with HIF-1α and responds to activating and inhibiting stimuli very similarly to HIF-1. It is now known not to be endothelial-specific, as once thought, but to be present in a number of cell types, and it is more frequently termed HIF-2α (24).

Hypoxia is common in all solid tumors (25) and also is associated with radiation resistance and poor prognosis (26). Therefore, the key upstream signal for VEGF regulation via the HIF-1α pathway is one target for therapies.

Here we demonstrate that hypoxia and degree of confluence are important regulators of VEGF in bladder cancer cell lines, and regulation differs in superficial versus invasive cell lines. This regulation is effected in part through the transcription factors HIF-1α and HIF-2α and in part through a PI 3-kinase-dependent pathway. We also demonstrate for the first time that both HIF-1α and HIF-2α are differentially expressed in human primary bladder tumors and related to VEGF mRNA expression. Having demonstrated previously the importance of VEGF in bladder tumors, this evidence suggests that the hypoxia response pathway is one in vivo mechanism regulating VEGF and may be a target for therapies.

MATERIALS AND METHODS

Cell Culture. The bladder carcinoma cell lines were a gift from Dr. Margaret Knowles (Imperial Cancer Research Fund, Leeds, United Kingdom). Cell lines were cultured in RPMI 1% medium (Clare Hall Laboratories, Imperial Cancer Research Fund) supplemented with 10% FBS and 4 mM glutamine and serially passaged in the laboratory using 0.25% trypsin-0.02% EDTA. Cells were routinely cultured in 95% O₂ and 5% CO₂ at 37°C and made hypoxic by placing them in a Heto-Holten Cell House (Heto-Holten, Camberley, Surrey, United Kingdom) at 0.1% O₂, 5% CO₂, and 94.9% nitrogen. The hypoxia pathway is also stimulated by CoCl₂ and DFO (30). The effect of these was assessed by changing complete medium to medium supplemented with either CoCl₂ (final concentration, 100 μM) or DFO (final concentration, 100 μM) and then routine culture for an additional 16 h. For PI 3-kinase inhibition experiments, LY 294002 (Sigma Chemical Co., Aldrich, Poole, Dorset, United Kingdom) was added to fresh serum-free medium immediately before 16 h of normoxia/hypoxia incubation at a final concentration of 20 μM. This concentration had no effect on the viability of our cells, whereas preliminary experiments increasing the dose (40 μM) of LY 294002 failed to produce a larger response and was toxic to our cells, with >10% reduction in cell counts (data not shown).

Measurement of VEGF Protein Levels in Cell Supernatant. Cells were plated in 1.5 ml of media/well in six-well culture plates (Falcon; Becton Dickinson Labware, Oxford, United Kingdom) at a density of between 1 × 10⁵ and 6 × 10⁵, depending on cell type and degree of confluence desired for the start of that experiment. At that stage, culture medium was aspirated and replaced with fresh assay medium. After 16 h of hypoxia (0.1%) or normoxia, medium was collected, centrifuged to remove cellular debris, and stored at −70°C until assayed for VEGF. VEGF assay was performed using a commercially available ELISA kit (R & D Systems, Abingdon Oxon, United Kingdom). Results between wells were standardized according to the cell number/well as measured on an automated Coulter counter (Beckman-Coulter, High Wycombe, Bucks, United Kingdom) and expressed as pg of VEGF protein/ml supernatant/10⁵ cells.

RNA Analysis. Total RNA was extracted from ~200 mg tissue using TRI reagent (Sigma Chemical Co., Aldrich, United Kingdom).

RNAse protection assays, using the method essentially as described by Petersen et al. (31), were carried out in duplicate on 10–30 μg of total RNA. [32P]CTP-labeled riboprobes for VEGF (6), HIF-1α (accession no. X95580) and HIF-2α (accession no. U81984) were prepared to the highest specific activity possible. To attenuate the signal strength of the highly abundant loading control, U6 small nuclear RNA (accession no. X01366), a riboprobe of significantly lower specific activity, was prepared (32).

The protected fragment size for VEGF₁₂₁ was 517 nt and for VEGF₁₆₅ and VEGF₁₈₉ was 439 nt, thus giving two bands on autoradiographs. Those for HIF-1α, HIF-2α, and the loading control were 206, 210, and 106 nt, respectively. On each gel a positive and negative (RNA only) control and undigested riboprobes were run. Intensity of signal, quantified on a phosphor-imager (Molecular Dynamics, Sunnyvale, CA), was calculated as the ratio of signal of interest/U6 signal to account for minor loading differences.

Protein Extraction and Immunoblot Analysis. For whole-cell extracts, cells were washed with ice-cold phosphate-buffered saline and collected by scraping. Cell pellets, or tumor samples, were homogenized in extraction buffer [8 mM urea, 10% glycerol, 10 mM Tris-Cl (pH 6.8), 1% SDS, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin] using an IKA Ultra-Turrax T8 homogenizer (Janke & Kunkel, Staufen, Germany) for 15 s at full speed. Extracts were quantified using the Pierce BCA protein assay (Pierce, Rockford, IL).

For immunoblotting, proteins were separated in SDS 6% polyacrylamide gels and transferred with a semidry blotter Imm-2 (W.E.P. Company, Concord, CA) to Immobilon P membrane (Millipore, Bedford, MA) overnight in 25 mM Tris base, 190 mM glycine, and 15% methanol. Membranes were blocked with 5% fat-free milk, 0.05% Tween 20, in phosphate-buffered saline. For HIF-1α detection, MoAb 28b (21) was used at

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4 μg/ml, and for HIF-2α, MoAb 190b (21) was used at 2 μg/ml. Detection was performed with horseradish peroxidase-conjugated goat antiamouse Immunoglobulins (Dako, High Wycombe, United Kingdom) at 1:1000 and ECL (Amersham Corp., Bucks, United Kingdom). After exposure, membranes were stained with Ponceau S to verify equal protein loading and transfer. Analysis of protein expression was performed by densitometry (Flurchemp; Alpha Innotech Corp., San Leandro, CA).

Tumor Samples. Superficial transitional cell bladder cancer was obtained from patients undergoing transurethral resection of bladder tumor, and invasive transitional cell bladder cancer and normal bladder was obtained from patients undergoing cystectomy at the Churchill Hospital. Immediately after resection, all samples were snap-frozen in liquid nitrogen. After patient histology confirmed the diagnosis, two 200- to 400-mg portions were removed from the stored sample on ice and used for mRNA and protein preparation.

Statistics. Statistical analysis was performed using Excel (Microsoft). Comparisons between groups were made using Student’s unpaired t test. The level of significance was P < 0.05.

RESULTS

Analysis of VEGF Protein and mRNA Expression by Bladder Cancer Cell Lines and the Effect of Hypoxia and Confluence. The highest constitutive expression of VEGF protein under standard cell culture conditions (normoxia and exponential growth phase) was seen in the superficial cell line RT4. Hypoxia up-regulated VEGF protein expression in RT4, 253J, and EJ28 (P = <0.01) but not in RT112 (P = 0.3) during exponential growth (Fig. 1).

Confluence also had an effect on basal VEGF protein expression, significantly reducing it in RT4, 253J, and EJ28 (P = <0.05) but not in RT112 in normoxia. This effect was standardized for cell number, indicating that, on average, each individual cell produced less VEGF.

When the effects of confluence and hypoxia were examined together, confluence abolished hypoxia-induced VEGF up-regulation in the superficial cell line RT4, but did not affect hypoxic induction in the invasive cell lines (253J, P = <0.01; EJ28, P = <0.05). Furthermore, confluence induced hypoxic up-regulation of VEGF in RT112 not seen during exponential growth (P = <0.05). Thus, whereas exponentially the most superficial cell line expressed the most VEGF, at confluence, the invasive cell lines, by virtue of greater hypoxic induction, expressed more. Thus, in three of four cell lines, confluence reduced basal VEGF, but only in the superficial cell line RT4 did confluence abolish hypoxic inducibility.

This suppression of VEGF induction at confluence in the superficial cell line RT4, but not the invasive cell line EJ28, was also seen when cultures were treated with either CoCl2 (100 μM, final concentration) or DFO (100 μM, final concentration), chemical mimics of hypoxia that induce this pathway (Fig. 2).

The same relative pattern of hypoxic induction of VEGF was reflected at the mRNA level (Fig. 3, A and B). To elucidate further the pathways regulating mRNA levels, we analyzed expression of HIF-1α and HIF-2α in normoxia and hypoxia and under different confluence conditions.
strong hypoxic up-regulation of both HIF-1α and HIF-2α, and the degree of hypoxic induction during both exponential and confluence growth phases was more similar than in RT4 (The fold inducibility of HIF-1α and HIF-2α progressing from normoxia to hypoxia at confluence was 60% and 50% for 253J and 74% and 61% for EJ28, respectively, that of hypoxic induction seen during exponential growth). Thus, at confluence, maintenance of hypoxic up-regulation of HIF-1α and, to some degree, HIF-2α corresponded with maintained VEGF inducibility; whereas in RT4 the loss of hypoxic VEGF inducibility corresponded with a reduction in HIF-1α and HIF-2α inducibility.

RT112 expressed very low levels of HIF-2α, both basally and under hypoxia, but there was strong hypoxic induction of HIF-1α, which was equal under exponential or confluence conditions. This HIF-1α up-regulation was not associated with an increase in VEGF expression during exponential growth but was associated with an increase in VEGF expression at confluence. The findings at the protein level for HIF-1α and HIF-2α expression were not seen at the mRNA level, where there was no difference between normoxic and hypoxic levels (data not shown).

Relation of PI 3-kinase to Hypoxic Regulation of VEGF.
Confluence altered VEGF expression, and PI 3-kinase activity can be regulated by confluence and proliferation. Mazure et al. (33) showed in transient reporter assays that hypoxic regulation of hypoxia response element constructs was mediated by PI 3-kinase. Therefore, we used the PI 3-kinase inhibitor LY 294002 to analyze interactions of hypoxia and confluence with HIF-1α and VEGF.

During exponential growth, LY 294002 caused a reduction in the expression of basal and, to a lesser degree, hypoxia-induced VEGF in all cases (Fig. 5A, Table 1). However despite a reduction in the expression of total VEGF, the inducibility produced by hypoxia was maintained or even increased in the presence of LY 294002. Indeed at confluence, whereas reduc-
A HIF-1α

RT4 | Nor | Nor | Hypo | Hypo | Exp | Conf | Exp | Conf
RT112 | Nor | Nor | Hypo | Hypo | Exp | Conf | Exp | Conf
253J | Nor | Nor | Hypo | Hypo | Exp | Conf | Exp | Conf
EJ28 | Nor | Nor | Hypo | Hypo | Exp | Conf | Exp | Conf

B HIF-2α

RT4 | Nor | Nor | Hypo | Hypo | Exp | Conf | Exp | Conf
RT112 | Nor | Nor | Hypo | Hypo | Exp | Conf | Exp | Conf
253J | Nor | Nor | Hypo | Hypo | Exp | Conf | Exp | Conf
EJ28 | Nor | Nor | Hypo | Hypo | Exp | Conf | Exp | Conf

Fig. 4 Hypoxia results in increased protein expression of HIF-1α and HIF-2α (three of four cell lines). Whereas this induction is relatively maintained in the invasive cell lines (253J and EJ28) at confluence, there is marked loss of induction in superficial cell line RT4. Cells were grown under routine cell culture conditions to either exponential or confluent growth phases. Medium was then replaced before 16 h of additional incubation under one of the following conditions: exponential growth phase/normoxia; exponential growth phase/hypoxia (0.1% O₂); confluent growth phase/normoxia; or confluent growth phase/hypoxia (0.1% O₂). Then cells were harvested rapidly by scraping on ice and homogenized in 8 M urea. One hundred μg of total protein of each sample was separated by SDS/PAGE (6% gel), and transferred to Immobilon-P. HIF-1α and HIF-2α were detected using MoAbs 28b and 190b respectively, goat antimouse secondary antibody, and ECL. Samples were run in duplicates. Representative blot shown of experiment repeated twice.

One hundred m of total protein was expressed more in invasive bladder (Fig. 7). HIF-1α and HIF-2α protein in tumor specimens compared with normal tissue was unaffected by the presence of LY 294002 (Fig. 6).

Analysis of HIF-1α and HIF-2α Expression in Bladder Cancers. To analyze whether, in freshly collected tumor samples, HIF-1α or HIF-2α was up-regulated and the mechanism of up-regulation, HIF-1α or HIF-2α protein and mRNA were measured in 4 normal and 12 bladder cancer cases (6 superficial and 6 invasive). There was increased expression of both HIF-1α and HIF-2α protein in tumor specimens compared with normal bladder (Fig. 7). HIF-1α protein was expressed more in invasive tumors than in superficial, although there was significant intertumor variation, with the highest expression actually in one of the superficial tumors (S5). HIF-2α protein expression was greatest in superficial tumors, and those tumors that had high HIF-2α expression tended to have high HIF-1α coexpression.

High mRNA expression of one factor did not correlate with high expression of the other (Fig. 8), nor was there an obvious association between mRNA levels and protein levels of either HIF-1α or HIF-2α. This is consistent with the main regulation of these factors being posttranslational oxygen-mediated degradation. Nevertheless, mRNA was much higher in some tumors (S4, S6, I1, and I2) for either HIF-1α and/or HIF-2α (with equal loading), raising the possibility of oncogene-induced overexpression (34). A significant correlation existed between VEGF mRNA and HIF-1α mRNA (Spearman rank correlation, r = 0.68; P = 0.02), raising the possibility of an oncogene or stress response up-regulating both VEGF and HIF-1α mRNA.

DISCUSSION

In this study, we have analyzed the effect and mechanism of the regulation of hypoxic response of VEGF in bladder cancer cell lines. We chose cell lines considered to represent a spectrum from superficial to invasive bladder cancer, because previously we showed two pathways regulating angiogenesis, VEGF more prominent in superficial tumors, and thymidine phosphorylase more prominent in invasive tumors. First, we analyzed the effect of hypoxia. Hypoxic tumors present therapeutic difficulties partly attributable to enhanced radioresistance and partly because of poor tissue penetration and the activity of certain chemotherapeutic agents in hypoxic conditions (26). Now it is clear that hypoxia per se triggers mechanisms including angiogenesis which may give tumors additional survival advantages. The regulation of VEGF by hypoxia was variable between cell lines, but all cells showed inducibility under at least one experimental condition. This is consistent with our previous findings of cell specificity in hypoxic response, where in breast carcinoma cell lines there was a 1.4- to 6.9-fold range of VEGF induction (35).

Contrary to other studies that have shown increased VEGF with increasing cell density in human glioblastoma (U87), fibrosarcoma, (HT1080), and colon carcinoma cell lines (36, 37), we demonstrated decreased VEGF protein expression in three of four bladder cancer cell lines at confluence under both normoxic and hypoxic conditions when compared with exponential growth. Cells enter a quiescent phase at confluence, with
changes in the expression of various cell-surface receptors, transcription factors, and growth factors (38). Basic fibroblast growth factor, another key angiogenic factor, was also reduced at confluence in renal cancer cell lines (39), although the mechanism is unknown.

Confluence may produce stresses, such as low glucose and acidosis, that contribute to VEGF regulation. Because the toxicity of hypoxia is proportional to cell density, with nutritional deprivation and acidosis contributing to hypoxia-triggered apoptosis (40), the combination of hypoxia and confluence was also examined. A loss of hypoxic inducibility of VEGF at confluence in the superficial cell line RT4, but not in the invasive cell lines 253J and EJ28, and indeed the gain in RT112, suggests that these latter cells may have a malignant growth advantage. Most superficial bladder tumors have a typical papillary structure, increasing the tumor surface area, and this may result in less tumor cell crowding with the preservation of high VEGF expression in these tumors, as we have demonstrated previously (6). Invasive tumors are solid, with associated increased cell density and intratumoral hypoxia, but adaptive mechanisms we have described in vitro may ensure that VEGF induction is maintained. Our results analyzing protein expression of HIF-1α and HIF-2α can explain some, but not all, of these findings.

Increased HIF-1α protein generally correlated with increased VEGF expression. One exception to this was RT4, where hypoxic up-regulation of VEGF was lost at confluence. Here, however, the fold induction of HIF-1α going from normoxia to hypoxia was much less at confluence than during exponential growth (Table 1). For 253J and EJ28, where this fold hypoxic induction of HIF-1α was maintained exponentially and at confluence, VEGF inducibility was also maintained. The results for RT112 are harder to explain, because an equal fold induction of HIF-1α under hypoxia is not translated to increased VEGF expression exponentially but occurs only at confluence. These results suggest that hypoxic and density-dependent regulation of VEGF is a balance between HIF-1α up-regulation...
interacting with other factors regulated at confluence (41). In addition, RT112, the only cell line to show hypoxic induction at confluence but not exponentially, was also the only cell line without HIF-2α. Whether HIF-2α has a specific role in induction under exponential growth will require additional investigation.

The increase in HIF-1α and HIF-2α at confluence in normoxia, seen most strikingly in RT4, but also in 253J and EJ28, may be attributable to mild hypoxia from cell-to-cell contact. Normoxic expression of HIF-1α has only rarely been reported previously (42). Integrins mediating cell-to-cell contact also use the PI 3-kinase/AKT pathway via integrin-linked kinase (43). Because of these observations and ours that confluence at normoxia (as measured by gas input) up-regulates HIF-1α and HIF-2α but does not increase VEGF, signaling pathways modified by confluence were analyzed. PI 3-kinase activates protein kinase B/AKT and mediates signaling for various growth factors, cytokines, and cell adhesion molecules (44). The suggestion that at least two pathways may interact to regulate VEGF expression under hypoxia and confluence is supported by our results with the inhibitor of the PI 3-kinase signaling pathway, LY 294002. This reduced total VEGF expression but still allowed hypoxia inducibility. HIF-1α protein induction was also unaffected by PI 3-kinase inhibition, implying that hypoxia inducibility of VEGF is conferred via HIF-1α but is additive to a basal pathway regulated substantially by PI 3-kinase. This result differs from results of Mazure et al. (33), who, using transient transfection of hypoxia response elements into Ha-Ras-transfected NIH3T3R cells and Rat-1 Ras transformed cells, demonstrated significant reduction of VEGF promotor activity under hypoxia with another inhibitor of PI 3-kinase (Wortmannin). They found no effect on basal levels of VEGF promotor activity. In addition, overexpression of a dominant negative mutant of the p85 regulatory subunit of PI 3-kinase was independent of this. The results on these bladder cell lines differ from two recent papers showing that PI3 kinase modulates HIF-2 promoter induction under hypoxia. They concluded that PI 3-kinase mediated VEGF induction through hypoxia response elements. Our results support the findings of Arbiser et al. (45) that, whereas increased expression of VEGF occurs through cellular PI 3-kinase activity, hypoxic regulation is independent of this. The results on these bladder cell lines differ from two recent papers showing that PI3 kinase modulation, either by expression of PTEN or PI3 kinase inhibitors,
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Data suggest that although HIF-1α and HIF-2α are both upregulated in all tumors. This is the first demonstration of up-regulation of these pathways in human bladder tumors. However, as in the cell lines, there was significant individual variation. Our analysis of human primary tumors revealed greater HIF-1α and HIF-2α protein expression in tumors compared with normal bladder specimens with one or other factor elevated in all tumors. This is the first demonstration of up-regulation of these pathways in human bladder tumors. However, as in the cell lines, there was significant individual variation. Our in vitro data suggest that although HIF-1α and HIF-2α are key components of hypoxic regulation of VEGF, clearly other transcription factors are also important in controlling angiogenesis. As such, and combined with our clinical data, therapy targeting these elements may be of benefit. Variable interactions with apoptotic pathways, angiogenic factors, and other growth factors may explain why experimentally some studies of antagonism of these pathways, angiogenic factors, and other growth factors may explain why experimentally some studies of antagonism of these pathways in bladder cancer compared with normal bladder specimens. Total RNA was prepared from specimens using Tri reagent (Sigma Chemical Co.) and mRNA was quantified by RNase protection assay (A). Intensity of signal is quantified, and displayed graphically in B, by dividing the intensity of signal of interest by the intensity of signal of loading control. U6, small nuclear RNA.

ACKNOWLEDGMENTS

We thank Dr. Kate Talks for advice using HIF-1α and HIF-2α antibodies.

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*Clin Cancer Res* 2001;7:1263-1272.

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