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Hypoxia Prolongs Monocyte/Macrophage Survival and Enhanced Glycolysis Is Associated with Their Maturation under Aerobic Conditions

John Roiniotis, Hang Dinh, Paul Masendycz, Amanda Turner, Caryn L. Elsegood, Glen M. Scholz, and John A. Hamilton

In chronic inflammatory lesions macrophages are abundant and adapt to the low oxygen concentrations often present there. In low oxygen some cell types die by apoptosis, as reported for macrophage cell lines, while others survive better as they shift their metabolism to anaerobic glycolysis. It was found here that hypoxia prolongs the survival of murine bone marrow-derived macrophages, either in the absence or presence of low CSF-1 (M-CSF) concentrations. Although Akt activity increased in bone marrow-derived macrophages in the low oxygen conditions, the levels of both anti- and proapoptotic Bcl-2 family members decreased. Glycolysis was enhanced as judged by increased glucose uptake, glucose transporter expression, lactate dehydrogenase mRNA expression, and lactate secretion. Human monocytes responded similarly to low oxygen, and a number of genes associated with glycolysis were shown by microarray analysis and quantitative PCR to be up-regulated. Interestingly, human monocyte-derived macrophages showed evidence of enhanced glycolysis even under aerobic conditions. It is proposed that certain monocyte/macrophage populations survive better under conditions of low oxygen, thereby contributing to their increased numbers at sites of chronic inflammation and tumors; it is also proposed that as macrophages differentiate from monocytes they begin to adopt a glycolytic metabolism allowing them to adapt readily when exposed to low oxygen conditions. The Journal of Immunology, 2009, 182: 7974–7981.

Macrophage populations depend for their development on CSF-1 (M-CSF) and often undergo apoptosis in its absence (1, 2). Very few studies have addressed the role that metabolic changes play in meeting the energy demands of their survival. It has been shown previously that prosurvival and mitogenic factors for macrophages, such as CSF-1 and GM-CSF, can stimulate macrophage glucose uptake with possible relevance to their survival and proliferation (3, 4). Macrophages are a prominent cell type found in increased numbers at sites of chronic inflammation, solid tumors, and immune reactions as part of the host reaction to injury and infection (5, 6); this increase in numbers can correlate with poor disease prognosis, and a major contribution to this increase most likely results from enhanced trafficking of blood monocytes into the infected tissue. Unless provided with an appropriate stimulus, inflammatory macrophages and neutrophils die by apoptosis, providing a potential mechanism for the resolution of inflammation (7, 8); therefore, there could also be a contribution to the increased macrophage numbers from enhanced survival within the inflamed tissue, due to the action of prosurvival stimuli in reversing apoptosis (8). CSF-1 and GM-CSF are two such potential stimuli since they have been found at elevated levels at sites of inflammation and ischemia and have been considered to have proinflammatory activities in part because of this prosurvival function (9, 10).

Reduced blood supply to sites of inflammation or damage and to malignant lesions can lead to reduced oxygen tension to levels <1% of those found in healthy tissues (11, 12). Among other changes occurring at these hypoxic sites, for example, the rheumatoid synovium (5), are the increased numbers of macrophages that appear to be capable of adapting to the reduced oxygen levels. Macrophages can function well under hypoxic conditions (13), and a number of associated cellular changes have been reported to occur, such as morphology, surface marker expression, phagocytosis, and migration (11, 12, 14). Also, in vivo in the steady-state monocytes/macrophages reside in areas of widely varying oxygen tensions, such as the blood, lung, and peritoneum, again indicating their necessary adaptability in response to this parameter (15).

Under normoxic conditions, cells usually metabolize glucose to CO₂ and water in an oxygen-dependent and highly energy-efficient manner with the production of 38 ATP molecules per glucose molecule (16, 17). Metabolism mainly involves glucose conversion to pyruvate followed by pyruvate catabolism through the TCA cycle and oxidative phosphorylation in the mitochondria. In contrast, glycolysis involves the metabolism of glucose via pyruvate to lactic acid and is less energy efficient, producing only two ATP molecules per glucose molecule (16, 17). Conversion of glucose to lactic acid in the presence of oxygen is known as aerobic glycolysis or the “Warburg effect” and is a feature of many tumors (16). Cells adapt to low oxygen tension by switching from an aerobic to an anaerobic glycolytic pathway for ATP production. For many cell types a major effect of hypoxia is apoptosis (18–21) and is the response so far reported in macrophage cell lines (22–25).
However, enhanced survival due to hypoxia has also been noted for other cell types (26–28). Candidate intracellular regulators and/or pathways governing the hypoxia-dependent survival/apoptosis balance in various cell types include the hypoxia inducible transcription factor-1α (29), glycolysis (30), Akt activity (27, 30, 31), Bcl-2 family members (27), and the transcription factor NF-κB (28).

Despite the findings mentioned above showing that macrophage cell lines die by apoptosis when exposed to a hypoxic environment (22–25), we reasoned that hypoxia might promote survival of primary macrophages especially since, as mentioned, certain macrophage populations can adapt well to these conditions (13) and since it is important where possible to use primary cell populations rather than cell lines. Using an IL-3-dependent myeloid cell line it was recently concluded that growth factor availability is critical for maintaining cell viability during chronic hypoxia and that anaerobic glycolysis is an effective hypoxia-induced survival strategy only under conditions of high intracellular glucose availability (32). However, since CSF-1 and GM-CSF have been shown previously to stimulate glucose uptake in macrophages (3, 4), we wondered whether hypoxia may do likewise and thereby also enhance macrophage survival. We report herein that exposure of primary murine macrophages and human peripheral blood monocytes to hypoxia (1% O2) can prolong the survival of monocytes/macrophages in growth factor-deprived populations most likely by enhancing glycolysis; we also show that as human monocytes differentiate in vitro into macrophages, they begin to acquire a glycolytic metabolism with possible significance for the ability of tissue macrophages to adapt to hypoxia.

Materials and Methods

Mice

Bone marrow cells were obtained from the following mice. As wild-type controls, C57BL/6 mice, originally obtained from Central Animal Services, Monash University (Clayton, Victoria, Australia), were routinely used. GM-CSF gene-deficient (GM-CSF−/−) mice, backcrossed onto the C57BL/6 background for 12 generations, were originally provided by the Ludwig Institute for Cancer Research (Parkville, Victoria, Australia). Csf1op2Csf1op2 mice (33) were also obtained from the Ludwig Institute.

Reagents

Reagents used were as follows. Cell culture medium and supplements, Glut-Max-1 and PenStrep, oligo(dT), RNaseOUT, and Tris-glucose SDS-PAGE gels (Invitrogen); HEPES (BDH Laboratory Supplies); PBS (CSL); recombinant CSF-1 (Chiron) and GM-CSF (PeproTech); RNase-free Mini-prip kit; Omniscript RT kit, and RNase-free DNase (Qiagen); SYBR Green and Universal Master Mix and predeveloped TaqMan probe/primer sets (Applied Biosystems); succinate dehydrogenase complex, subunit A (SDHA) and TATA box binding protein (TBP) primers (GeneWorks); protein assay dye reagent (Bio-Rad); lactate reagent solution and lactate standards (SDHA) and TATA box binding protein (TBP) primers (GeneWorks); predeveloped lactate reagent solution and lactate standard (SDHA) and TATA box binding protein (TBP) primers (GeneWorks); pro- and Universal Master Mix and predeveloped TaqMan probe/primer sets (Applied Biosystems); AmpliTaq Gold 360 Master Mix, AmpliTaq Gold 360 Q-PCR Master Mix, and AmpliTaq Gold 360 Q-PCR Master Mix (Life Technologies) according to the manufacturer’s protocol.

Preparation of bone marrow-derived macrophages (BMM)

Adherent murine macrophage populations were generated from precursors in the presence of CSF-1 (BMM) or GM-CSF (GM-BMM), as previously described (34, 35). Briefly, bone marrow cells were isolated from the femurs of mice and cultured in RPMI 1640 media (Invitrogen), supplemented with penicillin (100 U/ml)/streptomycin (100 U/ml), 20 mM HEPES (BDH Laboratory Supplies), and 10% heat-inactivated FBS in the presence of 5000 U/ml of M-CSF or GM-CSF in tissue culture wells (Chiron and PeproTech, respectively). After a total of 7 days, adherent monolayers were harvested. For the experiments monitoring viable cell numbers over time, the cells were seeded into 6-well Iwaki plates at a density of 5 × 10⁵ cells/well in the presence of CSF-1 (or GM-CSF) and incubated overnight under normoxic conditions to allow for adherence. Following washing, the viable cell number was counted as the value at time = 0.

Purification of peripheral blood monocytes

Monocytes were isolated from buffy coats obtained from the Australian Red Cross Blood Bank. Briefly, the contents of one buffy coat were diluted with an equal volume of PBS and then layered over Ficoll-Paque. The tubes were centrifuged for 20 min at 2500 rpm at room temperature and the PBMC at the Ficoll interface were collected. Monocytes were then purified from the PBMC using the StemSep CD14+ selection kit ( Stem Cell Technologies) according to the manufacturer’s protocol.

Cell culture media

Cell viability was measured by the trypan blue exclusion method. Cells were removed from tissue culture plates using either cell dissociation buffer (Sigma-Aldrich) as per the manufacturer’s instructions or by incubating cells with PBS at 37°C for 20 min followed by gentle pipetting to dislodge cells.

Hypoxic treatment

Cells treated with hypoxia were transferred from incubation under normoxic conditions (21% O2 and 5% CO2) into a precalibrated hypoxic chamber (Coy Laboratories) with a 1% O2 and 5% CO2 flow for the indicated time periods.

Cell lysis, SDS-PAGE, and Western blotting

Cells were lysed directly in tissue culture dishes with either cell dissociation buffer (Sigma-Aldrich) as per the manufacturer’s instructions or by incubating cells with PBS at 37°C for 20 min followed by gentle pipetting to dislodge cells.

Quantitative PCR (qPCR)

Total RNA was prepared from 1 to 3 × 10⁶ cells using the Qiagen RNeasy Mini kit with on-column DNase I digestion, according to the manufacturer’s instructions. Purified RNA was quantified by UV spectrometry at 260 nm, and 1 µg was reverse transcribed using the Omniscript RT kit in conjunction with an oligo(dT) primer and RNaseOUT as per the manufacturer’s instructions.

Gene expression analysis was achieved by qPCR using the Applied Biosystems Prism 7900HT sequence detection system with SDS version 2.3. SYBR Green Master Mix was used with housekeeping genes TBP and SDHA. Primer sequences were: forward SDHA, TGGGAACAA-GAGGGGCATCTG, reverse SDHA, CCACACCTGCATCAAATTCATG; forward TBP, CAGGAAACCAGCAGCTAGT, reverse TBP, TTTCTTGGCTGAGCAGTCCGGAC (37); they were synthesized by Gene-Works. Universal Master Mix was used with predeveloped TaqMan probe/primer sets for selected genes of interest: Glut1 and lactate dehydrogenase (LDH) A (human/mouse), HK (hexokinase-2) (human), and PGK (phosphoglycerate kinase-1) (human).

Antibodies

Abs with the following specificities were used as listed. Actin (Santa Cruz Biotechnology); Bcl-2, Bcl-xL, Akt, and phospho-Akt (Ser473) (Cell Signaling Technology); Bad and Bax (BD Biosciences); glucose transporter (Glut)1 (a gift from D. James, Garvan Institute); and mouse IgG-HRP and rabbit IgG-HRP (Dako).

Abbreviations used in this paper: DOG, deoxyglucose; BMM, bone marrow-derived macrophages; Glut, glucose transporter; HK, hexokinase; LDH, lactate dehydrogenase; MDM, macrophage-derived macrophage; PGK, phosphoglycerate kinase; qPCR, quantitative PCR.
Glucose uptake

Glucose uptake was measured using the incorporation of the [3H]-labeled, nonmetabolizable glucose analog 2-DG (3, 4). Following a particular treatment, cells were washed in PBS at room temperature. One milliliter of prewarmed assay buffer (20 mM HEPES (pH 7.4), 135 mM NaCl, 5 mM KC1, 1.8 mM CaCl2, and plus 0.1 mM 2-DG (final concentration) was added to each well, followed by the addition of a further 50 μl of assay buffer containing 1 μl of [3H]-2-DG (8 Ci/mmol), and cells were incubated at 37°C for 3 min. The culture wells were placed on ice and washed extensively with ice-cold assay buffer containing 5.5 mM d-glucose until no radioactivity was present in the washings. Cells were then dissolved with 0.1 M NaOH/0.1% Triton X-100 and counted using Scintiscint scintillation cocktail and a Beckman LS 6500 beta counter. The protein concentrations of the lysates were determined using the BCA protein reagent, and the results were expressed on a per μg protein basis.

Lactate secretion

The quantitative enzymatic determination of secreted lactate in conditioned media was determined using the Trinity Biotech lactate reagent as per the manufacturer’s instructions.

Microarray analysis

Microarray experiments were performed following recommended protocols supplied by Agilent Technologies. RNA was independently isolated from four donors, and RNA integrity was tested by electrophoresis (Bioanalyzer 2100; Agilent Technologies). RNA (800 ng) was amplified using the Low RNA Input Linear Amplification Kit (Agilent Technologies). The labeled cRNA was fragmented/hybridized to an Agilent Technologies whole human genome microarray containing 43,376 probes corresponding to 50,000 human genes (1). Once again when a low CSF-1 concentration is presumably due to the removal of CSF-1 by the BMM as has been described (38). It was observed that under hypoxic conditions the macrophages became less adherent and less spread on the tissue culture surface even though remaining viable. This relative difference in BMM survival was also maintained over a longer time period (see Fig. 2).

Hypoxia-enhanced BMM survival does not require endogenous CSF-1 or GM-CSF. One potential mechanism for this enhanced BMM survival in hypoxia is increased production of endogenous CSF-1 and/or GM-CSF. This explanation does not seem to be the case since BMM from both Csf1op/Csf1op and GM-CSF knockout mice also survive better and to a similar extent under hypoxic conditions as the BMM from C57BL/6 mice (Fig. 2A–C) (the Csf1op/Csf1op mice have a spontaneous inactivating mutation in the Csf1 gene (1)). Once again when a low CSF-1 concentration (320 U/ml) was included, the difference between hypoxia and normoxia was still maintained (data not shown). It has been shown that hypoxia induces CXCR4 expression and function in macrophages as judged by surface expression and chemotactic responsiveness to its cognate ligand, CXCL12 (39). CXCL12 also can promote cancer cell survival. It is therefore possible that endogenous CXCL12 (SDF-1) may be contributing to the hypoxia-induced survival shown above. However, the CXCL12 antagonist AMD 3100 (100 nM and 1 μM) (40) did not inhibit the hypoxia-induced BMM survival over 48 h (data not shown) as might be expected if this mechanism were responsible. Supporting this conclusion, microarray analysis revealed that hypoxia in fact downregulated CXCL12 mRNA in CSF-1-starved BMM (data not shown). We also found no evidence for an endogenous prosurvival mediator in 24-h supernatants of hypoxic-treated BMM from C57BL/6 mice when tested back on CSF-1-depleted C57BL/6 BMM (data not shown).

**Results**

**Hypoxia enhances BMM survival**

Hypoxia-enhanced BMM survival in the absence and presence of suboptimal CSF-1. BMM gradually die by apoptosis under “normoxic” conditions when CSF-1 is removed (1, 2). The data after 2 day CSF-1 removal can be seen in Fig. 1. When CSF-1 removal was conducted under hypoxic conditions, there were more viable cells remaining than during normoxia (68% vs 38%; p < 0.05). This difference was also manifested when increasing concentrations of CSF-1 are included that promote BMM survival under normoxic conditions (e.g., 80–640 U/ml) (3, 4) (Fig. 1). The reduced survival over time even in the presence of these low CSF-1 concentrations is presumably due to the removal of CSF-1 by the BMM as has been described (38). It was observed that under hypoxic conditions the macrophages became less adherent and less spread on the tissue culture surface even though remaining viable. This relative difference in BMM survival was also maintained over a longer time period (see Fig. 2).

Hypoxia-induced macrophage survival does not require endogenous CSF-1 or GM-CSF. One potential mechanism for this enhanced BMM survival in hypoxia is increased production of endogenous CSF-1 and/or GM-CSF. This explanation does not seem to be the case since BMM from both Csf1op/Csf1op and GM-CSF knockout mice also survive better and to a similar extent under hypoxic conditions as the BMM from C57BL/6 mice (Fig. 2A–C) (the Csf1op/Csf1op mice have a spontaneous inactivating mutation in the Csf1 gene (1)). Once again when a low CSF-1 concentration (320 U/ml) was included, the difference between hypoxia and normoxia was still maintained (data not shown). It has been shown that hypoxia induces CXCR4 expression and function in macrophages as judged by surface expression and chemotactic responsiveness to its cognate ligand, CXCL12 (39). CXCL12 also can promote cancer cell survival. It is therefore possible that endogenous CXCL12 (SDF-1) may be contributing to the hypoxia-induced survival shown above. However, the CXCL12 antagonist AMD 3100 (100 nM and 1 μM) (40) did not inhibit the hypoxia-induced BMM survival over 48 h (data not shown) as might be expected if this mechanism were responsible. Supporting this conclusion, microarray analysis revealed that hypoxia in fact downregulated CXCL12 mRNA in CSF-1-starved BMM (data not shown). We also found no evidence for an endogenous prosurvival mediator in 24-h supernatants of hypoxic-treated BMM from C57BL/6 mice when tested back on CSF-1-depleted C57BL/6 BMM (data not shown).
We also tested whether a different murine macrophage lineage population, namely cells grown out from bone marrow precursors in the presence of GM-CSF instead of CSF-1, that is, so-called GM-BMM (34, 35), could also survive better in hypoxic conditions. We see in Fig. 2D that GM-BMM behave like BMM in this regard, indicating that the hypoxic effect is not confined to a CSF-1-generated macrophage population.

The effect of hypoxia on Akt activity and Bcl-2 family members in BMM. Akt activity is widely implicated in the suppression of apoptotic cell death (27). We therefore assessed whether its activity might be stimulated in BMM following hypoxia exposure relative to its activity in aerobic conditions. Lysates were then analyzed by Western blot for (A) and (C) Akt activity (anti-pAkt) and total Akt, and for (B) and (D) proapoptotic (Bad and Bax) and antiapoptotic (Bcl-2 and Bcl-xL) proteins. Actin served as a loading control. Data are from a representative experiment, which was repeated five times.

BMM during hypoxia. As might be anticipated, in CSF-1-depleted BMM the levels of the proapoptotic proteins Bad and Bax decreased over a 24-h exposure to hypoxia relative to their levels under normoxic conditions (Fig. 3B); however, the levels of the antiapoptotic family members Bcl-2 and Bcl-xL were also lower following hypoxia. The lack of correlation of hypoxia-induced BMM survival with expression of the antiapoptotic Bcl-xL and Bcl-2 suggested that their expression is not controlling the hypoxia-mediated reduction in apoptosis in this system, a conclusion drawn also for hypoxia-mediated survival in human neutrophils (26) (see Discussion).

The CSF-1-depleted BMM die relatively rapidly under normoxic conditions (Fig. 1) and their relative hypoxia-driven survival may require enhanced Akt activity (Fig. 3A). However, we see in Fig. 3C that cycling BMM, that is, BMM in the presence of...
Hypoxia and monocytes/macrophages

Glucose transporter expression. Mechanisms responsible for increased glucose uptake, for example in response to a hypoxic stimulus, include elevated glucose transporter activity, translocation, and/or expression (31). In hemopoietic cells, Glut1 is the most widely expressed and studied of such transporters (44). Following exposure of CSF-1-deprived BMM and cycling BMM to low oxygen, Glut1 mRNA levels increased (Fig. 4B). Glut1 protein expression also rose. The data for cycling BMM are provided in Fig. 4C.

Anaerobic glycolysis. As mentioned, the fate of glucose after cellular uptake is dependent on the oxygenation state of the cell, with a well-oxygenated cell being able to convert one glucose molecule into 38 ATP molecules (aerobic glycolysis), while in a low oxygen environment only 2 molecules of ATP are generated along with 2 molecules of lactate (anaerobic glycolysis) (16, 17). In the same experiment whose Glut1 mRNA data were provided in Fig. 4B, mRNA expression of the glycolytic enzyme LDH also increased in a similar manner (Fig. 4D). We measured lactate secretion in cycling BMM and found that hypoxia increases it (Fig. 4E).

Hypoxia and human monocytes/macrophages

Hypoxia enhances human monocyte survival. Peripheral blood monocytes gradually die by apoptosis unless provided with a stimulus such as CSF-1 (42). We determined whether the prosurvival action of hypoxia on murine macrophage populations shown above extends to human monocytes. The data in Fig. 5A demonstrate that indeed it is the case. On the other hand, if the monocytes were cultured in CSF-1 for 10 days to differentiate them into adherent MDM (45), the cells did not die in the absence of CSF-1 for at least 3 days under aerobic conditions (see also Ref. 46) and hypoxia did not reduce this viability (data not shown).

Glucose uptake. We have shown previously that CSF-1, in a dose-dependent manner, as well as other prosurvival and mitogenic stimuli, can stimulate glucose uptake in CSF-1-starved BMM as measured by uptake of the nonmetabolizable analog 2-DG (3, 4); this glucose uptake has been shown to be essential for CSF-1-stimulated BMM survival (43). Both the basal 2-DG uptake in BMM and that induced by CSF-1 and a range of stimuli have previously been demonstrated to occur via a carrier-facilitated d-glucose transport system as judged by inhibition by cytochalasin B, phloretin, and high concentrations of n-glucose (3, 4). We therefore examined whether hypoxia can also enhance glucose uptake in the same cells. As seen in Fig. 4A, it was able to do so, as measured by [1H]2-DG uptake following 24 h of hypoxic treatment. The increased [1H]2-DG uptake following hypoxia was noted for both CSF-1-depleted and cycling BMM, with the latter already having higher values in normoxia as expected (3, 4) (Fig. 4A).

Table 1. Regulation of “glycolytic” genes in human monocytes and MDM by hypoxia

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>Mono (H)</th>
<th>MDM (N)</th>
<th>MDM (H)</th>
</tr>
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<tr>
<td>ALDOC</td>
<td>Aldolase C, fructose-bisphosphatase</td>
<td>5.7</td>
<td>6.5</td>
<td>13.0</td>
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<tr>
<td>PGAM2</td>
<td>Phosphoglycerate mutase 2</td>
<td>5.7</td>
<td>3.2</td>
<td>12.1</td>
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<td>ENO2</td>
<td>Enolase 2</td>
<td>4.6</td>
<td>3.0</td>
<td>7.5</td>
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<tr>
<td>GLUT1</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 1</td>
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<td>1.9</td>
<td>6.8</td>
</tr>
<tr>
<td>GPI</td>
<td>Glucose phosphate isomerase</td>
<td>3.5</td>
<td>3.2</td>
<td>4.9</td>
</tr>
<tr>
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<td>Enolase 1</td>
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</table>

a Microarray analysis was carried out on human monocytes (Mono) and MDM (n = 4 donors) that were subjected to normoxic (N) or hypoxic (H) conditions. Purified human monocytes and their MDM were cultured for 24 h in CSF-1 (5000 U/ml) under normoxic or hypoxic conditions. Data are mean values. Shown are genes in the ontological classification of glycolysis that are differentially expressed (fold change ≥2 and p ≤ 0.05) in hypoxic monocytes as compared to normoxic monocytes. Values are expressed as fold change when compared to the values for monocytes in normoxia, which were arbitrarily given a value of 1 for each gene.

FIGURE 5. The effects of hypoxia on human monocytes and macrophages. A. Purified human monocytes were seeded in 10% FBS and incubated for 3 days in triplicate cultures under normoxia or hypoxia (n = 5 donors). Viable cell number and expression as in Fig. 1. Data are mean values ± SEM from a representative experiment; p < 0.05, hypoxia vs normoxia. B. Purified human monocytes and (C) their MDMs were cultured in 10% FBS and [1H]2-DOG uptake measured in triplicate cultures following exposure to normoxia or hypoxia for 24 h. Data are mean values ± SEM from a representative donor (n = 3 donors); p < 0.05, hypoxia vs normoxia. D. Purified human monocytes and their MDM were cultured in triplicate in CSF-1 (5000 U/ml) in normoxia and hypoxia for 24 h in 10% FBS and Glut1 mRNA expression (qPCR) was measured. Data are mean values ± SEM from a representative experiment (n = 6 donors), with the normoxic values for the monocytes arbitrarily given a value of 1; p < 0.05, hypoxia vs normoxia.
also enhanced glucose uptake in both human monocytes (Fig. 5) and MDM the cell is beginning to adopt features of a glycolytic metabolism. To examine this possibility microarray analysis was conducted on the monocytes and MDM under normoxic and hypoxic conditions. In Table I, the “glycolytic” genes (Gene Ontology classification (47)), which are up-regulated ≥2-fold (p < 0.05) in hypoxic monocytes vs their counterparts under aerobic conditions, are listed. As for Glut1 mRNA (Fig. 5D), many of these up-regulated genes also showed an increased expression in normoxic MDM, relative to that in normoxic monocytes, with some even showing a larger change than in hypoxic monocytes. We next sought confirmation of these data by qPCR for some of the more widely studied genes of this class.

It can be seen that LDH mRNA (Fig. 6A) was elevated under normoxia to some extent in MDM compared with the monocytes; for both cell populations, LDH mRNA expression was enhanced further in hypoxia. Lactate secretion was elevated in hypoxic MDM compared with MDM under normoxic conditions when measured as an accumulation over the final 5-day period for MDM generation from the monocytes (see Materials and Methods) (Fig. 6B). When measured for monocytes over the short period of only 24 h there was no observable difference for monocytes when exposed to hypoxia. The qPCR data for other “glycolytic” enzymes, namely HK and PGK, are presented in Fig. 6, C and D, respectively. During differentiation from monocytes to MDM under normoxic conditions the glycolytic gene mRNAs, as for Glut1 (Fig. 5D) and LDH mRNA (Fig. 6A), were up-regulated. Likewise, in response to hypoxia, for both cell types HK and PGK mRNA expression rose.

**Discussion**

We have shown above for the first time that hypoxia can have a prosurvival effect on monocytes/macrophages. Prior studies, usually with murine macrophage cell lines, have reported hypoxia-induced apoptosis (22–25). We found that two populations of murine macrophages, as well as human monocytes, which all require a stimulus such as CSF-1 or GM-CSF for survival, demonstrated delayed apoptosis under hypoxic conditions in the absence of such a stimulus. For BMM, the enhanced survival was independent of endogenous CSF-1 or GM-CSF. We also observed that for BMM the prosurvival effects of hypoxia together with a low (i.e., suboptimal) CSF-1 concentration were more than for each stimulus alone, suggesting perhaps independence of the relevant pathways downstream of the two types of stimuli. Our findings of a prolongation of monocyte/macrophage survival by hypoxia are in contrast to what has been published for macrophage cell lines but are similar to what has been found with human neutrophils (26, 28).

Furthermore, they confirm the importance of having data from non-cell line systems and also highlight again the fact that hypoxia can have both prosurvival and proapoptotic consequences depending on the cellular context, with the particular response perhaps reflecting the ability of cells to adapt to anaerobic metabolism (28).

It was noted above that hypoxia stimulated Akt activity, which has been strongly implicated in cell survival in general, including that of monocytes/macrophages (27, 31, 48) and, of relevance to our findings, also linked to hypoxia-mediated survival in other cell types (20, 27) and to glycolysis (17, 27, 30, 49). It is therefore possible that pathways downstream of Akt are important for the hypoxia-stimulated monocyte/macrophage survival and glycolysis reported above. However, note that hypoxia can still up-regulate Akt in BMM that are in cycle, that is, are in the presence of CSF-1 (Fig. 3C); also, we found that the Akt inhibitor Akt VIII (10 μM) did not inhibit the hypoxia-stimulated BMM survival in the absence of CSF-1 over a 48-h period (data not shown). The down-regulation by hypoxia of the antiapoptotic Bcl-2 and Bcl-xL proteins, in both CSF-1-depleted and cycling BMM, is perhaps surprising given the literature on other cells (21) but could be due to suppression of overall protein synthesis reported during hypoxia for macrophages (50) and other cell types (51); consistent with this possibility, we found that mRNA levels of the two antiapoptotic proteins did not parallel the loss of protein (data not shown). In any case, Bcl-2 and Bcl-xL expression did not correlate with enhanced macrophage survival for CSF-1-depleted BMM; also, cycling BMM, that is, in the presence of a high CSF-1 concentration, whose survival is not modulated by hypoxia, also showed the same reduction. In this context, Bcl-2 was not considered to be involved in the prosurvival effect of hypoxia on human neutrophils (26). The Akt inhibitor also did not modulate Bcl-2 family protein expression in CSF-1-starved BMM under hypoxic conditions. Whether modulation of Bcl-xL deamination, which has been linked with apoptosis (52, 53), plays any role in the prosurvival effects of hypoxia on monocytes/macrophages described above is unknown.

During hypoxia it was observed that there was increased glucose uptake and Glut1 mRNA expression for BMM, and for human monocytes and MDM, with the increase in the latter parameter presumably contributing to that in the former. Others have shown...
that hypoxia can up-regulate macrophage expression of Glut1 with potential to increase the supply of intracellular glucose (13, 54). Enhanced lactate and/or LDH mRNA expression were also observed above as part of the hypoxic response for the monocytes and macrophage populations. The increased anaerobic glycolysis presumably contributes to the increased survival of the cell populations above that have insufficient intracellular glucose, such as CSF-1-depleted BMM. However, glucose uptake (and Akt activity) could still be noted in response to hypoxia for cells not undergoing apoptosis under normoxic conditions, such as CSF-1-treated BMM and human MDM (see below), suggesting that the enhanced ATP generated from the glycolytic pathway can be used for additional functions. Our observations on hypoxia and glycolysis with CSF-1-depleted BMM and GM-CSF-depleted GM-BMM would appear to be at odds with some of the findings with an IL-3-dependent myeloid cell line (32); in that study, hypoxia failed to up-regulate glycolysis and Glut1 levels when the cells were deprived of IL-3, leading the authors to conclude that anaerobic glycolysis is an effective hypoxia-induced survival strategy only under conditions of high intracellular glucose availability and that growth factor availability is critical for maintaining cell viability during chronic hypoxia. Our findings suggest that this concept is not a general one: it may apply only to chronic low oxygen exposure and needs to be confirmed with non-cell lines under similar long-term conditions.

One of the macrophage lineage populations examined above and that is widely used as an in vitro model for human macrophages, namely MDM, does not require an exogenous CSF for survival under aerobic conditions and therefore is not suitable to study any prosurvival activity of hypoxia (46, 55, 56). Others have found that hypoxia increased Glut1 mRNA levels in MDM and speculated that this change could be important for macrophage survival in hypoxic diseased tissue (54). In agreement with prior observations with a similar population (55, 56), we did not find a proapoptotic effect of hypoxia. As for the other monocyte/macrophage populations studied, glycolysis was promoted in MDM in the low oxygen concentration. However, in contrast to one of these reports (56), we found increased 2-DOG uptake for MDM in response to hypoxia (Fig. 5C); however, both studies found increased lactate production (Fig. 6B) (56). We noted at least at the gene level that, relative to the less differentiated monocytes isolated straight from blood, glycolytic enzymes were up-regulated in MDM to some extent even in normoxic conditions (Figs. 5D and 6 and Table I). It could be that this enhanced glycolysis in MDM vs monocytes forms part of a wider enhanced metabolic profile during maturation/differentiation as we and others have reported by microarray profiling for lipid, fatty acid, and carbohydrate metabolism (57, 58). The enhanced glycolytic gene expression in MDM appears to form part of the maturation differences from monocytes whereby they are beginning to be adapted to a glycolytic metabolism and thereby can respond rapidly to low oxygen exposure. This proposition is in line with the evidence that certain ex vivo myeloid populations (macrophages and neutrophils) are adapted to a hypoxic mode of existence by producing a significant proportion of their ATP through glycolysis (so called “aerobic glycolysis”) (13, 16). It is also supported by data for the human monocyte cell line THP-1, in which glycolysis is up-regulated following phorbol ester-induced macrophage differentiation, possibly via hypoxia inducible transcription factor-1α (59). It would be worthwhile to explore in more detail this concept at the biochemical level. Even though endogenous CSF-1, for example, may be responsible for MDM survival in aerobic conditions in the absence of an added stimulus (46), it is possible that the enhanced aerobic glycolysis observed in MDM may be contributing to this survival and would be consistent with the notion that a glycolytic phenotype can be associated with a state of apoptotic resistance (27).

As regards further the mechanism of hypoxia-induced cell survival (or reduced apoptosis), we found that certain genes associated with apoptosis, such as DAPK2 (death-associated protein kinase 2), FAS, and DNASE1, were down-regulated under hypoxic conditions in BMM and human monocytes (data not shown). As mentioned above, we also noted that under hypoxic conditions CSF-1-starved BMM become less adherent, with some cells detaching from the tissue culture surface. Whether the reduced adhesion is related to hypoxia-induced changes in monocyte/macrophage migration (60–62) is unknown. The microarray analysis for CSF-1-starved BMM showed reduced expression for certain cell adhesion molecules, for example, integrins, laminin, CD36, and protocadherins (data not shown). The significance of these gene changes for these two altered macrophage functions (i.e., survival and adhesion) awaits further experimentation.

With respect to the possible relevance of our findings to pathology, we suggest that hypoxia-induced monocyte/macrophage survival, via increased glycolysis, would be one means of increasing macrophage numbers in tumors and sites of inflammation by shifting the balance from apoptosis to survival and is one response of this cell lineage with its ability to adapt readily to anaerobic metabolism (12, 13, 54). Also, note that CSF-1 and GM-CSF are present at elevated levels in ischemic areas of diseases where macrophages have been found (9, 10, 12) and could be providing an additional prosurvival stimulus to hypoxia. As mentioned, hypoxia also inhibits neutrophil apoptosis (26, 28) and, based on our findings above, it is possible that at sites of low oxygen tension both macrophage and neutrophil longevity may be prolonged with implications for the rate of the resolution of inflammatory reactions.

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Disclosures
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References


