PTEN/Akt Signaling through Epidermal Growth Factor Receptor Is Prerequisite for Angiogenesis by Hepatocellular Carcinoma Cells That Is Susceptible to Inhibition by Gefitinib

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common tumor-related causes of death worldwide for which there is still no satisfactory treatment. We previously reported the antiangiogenic effect of gefitinib, a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that has been used successfully to treat lung cancer. In this study, we investigated the effects of gefitinib on tumor-induced angiogenesis by using HCC cell lines (HCC3, CBO12C3, and AD3) in vitro as well as in vivo. Oral administration of gefitinib inhibited angiogenesis induced by HCC3 and CBO12C3, but not by AD3 in the mouse dorsal air sac model. Production of both vascular endothelial growth factor (VEGF) and chemokine C-X-C motif ligand 1 (CXCL1) by EGF-stimulated HCC was more markedly inhibited by gefitinib in HCC3 and CBO12C3 cells than in AD3 cells. EGF stimulated the phosphorylation of EGFR, Akt, and extracellular signal-regulated kinase 1/2 (ERK1/2) in HCC3 and CBO12C3 cells, whereas EGF stimulated phosphorylation of EGFR and ERK1/2, but not Akt in AD3 cells. In fact, Akt was constitutively activated in the absence of EGF in AD3 cells. Gefitinib inhibited Akt phosphorylation in all three cell lines, but it was about five times less effective in AD3 cells. The concentration of PTEN in AD3 cells was about a half that in HCC3 and CBO12C3 cells. Transfection of HCC3 cells with PTEN small interfering RNA reduced their sensitivity to gefitinib in terms of its inhibitory effect on both Akt phosphorylation and the production of VEGF and CXCL1. In conclusion, effect of gefitinib on HCC-induced angiogenesis depends on its inhibition of the production of angiogenic factors, probably involving a PTEN/Akt signaling pathway.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide (1) and the prognosis for HCC patients is still very poor. Members of the epidermal growth factor receptor (EGFR) family have emerged as critical factors in the development and growth of various types of cancer, including HCC (2). These receptors are part of a complex network of signal transduction cascades that modulate tumorigenic processes, such as proliferation, adhesion, migration, differentiation, angiogenesis, and escape from apoptosis (3–6). High EGFR expression in human cancers is often correlated with advanced disease, metastasis, and poor clinical outcome, for example in non–small cell lung carcinoma (NSCLC), breast, cervical, and head and neck carcinomas (7–9).

Gefitinib (Iressa, ZD1839) is an orally active and selective EGFR tyrosine kinase inhibitor that blocks EGFR-mediated signal transduction pathways involved in cancer growth (10, 11). Gefitinib has antiproliferative activity in various human cancers in vitro as well as in vivo (12, 13). It is now approved as a monotherapy for patients with locally advanced or metastatic NSCLC (14–16) and is under investigation for the treatment of prostate, breast, head and neck, gastric, and colorectal cancer (17). An important recent discovery is the close association between a clinical response to gefitinib in patients with NSCLC and somatic mutations in the EGFR gene (18, 19). In NSCLC cells carrying such mutations, gefitinib treatment markedly inhibited phosphorylation of EGFR and its downstream signaling kinases, Akt and extracellular signal-regulated kinase 1/2 (ERK1/2). Consistent with this observation, we have reported that the sensitivity of NSCLC cell lines to gefitinib under basal growth condition is closely correlated with their dependence for survival and proliferation on Akt and ERK1/2 activation in response to EGFR signaling (20).

Cappuzzo et al. (21) have reported that patients with phosphorylated Akt–positive tumors who received gefitinib had a better response rate and time to progression. Moreover, increased EGFR gene copy number evaluated by fluorescent in situ hybridization (FISH) was significantly associated with higher response rates and lower progression rates in lung cancer (22) and in colon cancer (23).

HCC is a typical hypervascular tumor and tumor angiogenesis is a prerequisite for both its growth and metastasis (24, 25). Angiogenesis and vascular invasion are common characteristics of malignant tumors in patients with HCC (26, 27). Enhanced expression of vascular endothelial growth factor (VEGF) and chemokine C-X-C motif ligand 1 (CXCL1) is often seen both in HCC cells in culture and at sites of angiogenesis in the livers of HCC patients (26, 28–33). Ishikawa et al. (34) have previously reported antitumor effect by antiangiogenesis gene therapy using angiostatin gene on HCCs in the xenograft model. A recent study by Liu et al. (35) has also shown that administration of a VEGF tyrosine kinase inhibitor induced both antitumor and antiangiogenesis effects against HCCs in the xenograft model. On the other hand, we have previously reported the inhibition of EGF-induced migration of vascular endothelial cell and neovascularization in mouse corneas by gefitinib, suggesting that its antitumor effect...
PTEN gene silencing using small interfering RNA. The ability of 25-nucleotide duplexes of RNA to interfere with PTEN expression was tested. The specific small interfering RNA (siRNA) sequence used was nucleotide 58 to 82, relative to the first nucleotide of the start codon, of PTEN (Genbank accession number NM_008960). Mock siRNA duplexes were obtained from Invitrogen (Carlsbad, CA). siRNA duplexes were transfected using LipofectAMINE 2000 and Opti-MEM medium (Invitrogen) according to the recommendations of the manufacturer. Reduction in PTEN expression was confirmed by Western blot analysis.

FISH analysis. FISH analysis was done as described previously (43). Mouse EGFR cDNA (BC-023729-EGFR) and Mere Mouse BAC clone 43L24 were obtained from Open Biosystems (Huntsville, AL). The probes for EGFR and chromosome 11 were labeled with digoxigenin and biotin by nick translation methods, respectively. The slides were incubated at 75°C for 10 minutes to denature the EGFR and chromosome 11 probes and allowed to hybridize overnight at 37°C. After stringency wash, digoxigenin-labeled EGFR and biotin-labeled chromosome 11 probes were detected using antidigoxigenin-Cy3 and anti-biotin-Cy5. The chromatin was counterstained with 4',6-diamidino-2-phenylindole. Analysis was done with a Leica DMRA2 fluorescence microscope (Deerfield, IL) equipped with the Leica CW-4000 FISH software. Average numbers of chromosome were determined by scoring 30 to 40 metaphase spreads.

Statistical analysis. Statistical analysis used the Mann-Whitney U test and Student’s t test, with P < 0.05 considered statistically significant, and was done using JMP 5.01 software (SAS Institute, Inc. Cary, NC).

Results

Effect of EGF on the cell growth of HCC cell lines. The growth rates of HCC cell lines were measured under normal growth conditions with 10% FBS, in the presence or absence of 10 ng/mL EGF. Doubling times for HCC3, CB012C3, and AD3 were 13.5, 12.1, and 12.6 hours, respectively, in the presence of EGF and 22.4, 14.6, and 14.6 hours, respectively, in the absence of EGF (Fig. 1). Growth rates for the three lines were comparable, although EGF affected the growth of HCC3 cells rather more than that of the other two cell lines.

Gefitinib inhibits tumor-induced angiogenesis in vivo. We next investigated whether gefitinib could inhibit angiogenesis induced by HCC cells in vivo, using the dorsal air sac assay. In the absence of gefitinib, implantation of chambers containing each of the three HCC cell lines resulted in the development of microvessels with thin curled structures and tiny bleeding spots, in addition to the preexisting vessels (Fig. 2A), consistent with our previous studies (41). The oral administration of gefitinib at 150 mg/kg/d markedly reduced the development of microvessels induced by HCC3 and CB012C3 cells, but not by AD3 cells (Fig. 2A). About a 60% reduction in angiogenesis was seen in mice implanted with HCC3 cells and given 75 mg/kg/d gefitinib and a similar reduction was seen in mice implanted with CB012C3 cells and given 150 mg/kg/d gefitinib (Fig. 2B).

Inhibitory effect of gefitinib on production of VEGF and CXCL1 in HCC cells. To understand how gefitinib modulates angiogenesis, we examined the effect of this EGFR-selective drug on the production of two potent inducers of angiogenesis, VEGF and CXCL1 [interleukin-8 (IL-8) homologue/KC/Gro-α] in HCC cell lines (Fig. 3A). EGF increased the production of both VEGF and CXCL1 by 1.6- to 3.2-fold in all three cell lines. At 0.1 to 5.0 μmol/L, gefitinib inhibited EGF-stimulated production of VEGF and CXCL1 by HCC3, CB012C3, and AD3 to different extents. At 1 μmol/L, gefitinib blocked the production of CXCL1 by >80% in HCC3 and CB012C3 cells and by 60% in AD3 cells in the presence of EGF. At the same concentration, gefitinib also blocked VEGF production by
HCC3 and CBO12C3 cells by 60% to 80%, whereas VEGF production by AD3 cells was only inhibited by 20% in the presence of EGF (Fig. 3A). The production of VEGF and CXCL1 in response to exogenous EGF was rather more sensitive to inhibition by gefitinib in HCC3 and CBO12C3 cells compared with AD3 cells.

**Effect of gefitinib on phosphorylation of EGFR, Akt, and ERK1/2 in HCC cells.** When EGFR binds EGF or transforming growth factor-β, it is autophosphorylated and activates a number of downstream signaling molecules, such as Akt and mitogen-activated protein kinase (ERK1/2; ref. 42). We examined the effect of gefitinib on the phosphorylation of EGFR, Akt, and ERK1/2 in the three cell lines in vitro. Exogenous EGF enhanced the phosphorylation of EGFR, Akt, and ERK1/2 in HCC3 and CBO12C3 cells. By contrast, EGF enhanced the phosphorylation of EGFR and ERK1/2, but not Akt, in AD3 cells; Akt was constitutively phosphorylated in AD3 cells when EGF was added exogenously (Fig. 3B). Phosphorylation of Akt was also inhibited by gefitinib to different extents in the three cell lines: 0.1 μmol/L gefitinib inhibited Akt phosphorylation in HCC3 and CBO12C3 by 50% compared with EGF alone, but had no effect at this concentration in AD3 cells (Fig. 3B). Although Akt was constitutively phosphorylated in the absence of EGF in AD3 cells, phospho-Akt was rather less sensitive to the inhibitory effect of gefitinib in AD3 than in the other two cell lines.

**Involvement of Akt and ERK1/2 in EGF-induced production of angiogenic factors, VEGF and CXCL1.** To identify the EGF/EGFR-activated signal pathways involved in the production of angiogenic factors, we looked at the effects of LY294002, a selective inhibitor of PI3K, and U0126, an inhibitor of ERK1/2, on the production of VEGF and CXCL1 by HCC3 and AD3 cells. LY294002 inhibits Akt activation by specifically blocking PI3K, a positive regulator of Akt kinase. EGF increased VEGF and CXCL1 production in both these cell lines 2- to 3-fold (see Fig. 3A) and treatment with LY294002 resulted in a significant decrease in the production of both factors in HCC3 cells compared with EGF alone (Fig. 4A and B). However, LY294002, tested up to a concentration of 5 μmol/L, had no effect on the EGF-induced production of VEGF and CXCL1 in AD3 cells (Fig. 4A and B). Treatment with U0126 also affected cellular production of VEGF in both cell lines but there was no significant difference in the inhibitory effect of U0126 on cellular production of VEGF between two cell lines (Fig. 4C). U0126 showed only a slight, if any, inhibition of CXCL1 production, but there was no significant difference in its inhibition between two cell lines (Fig. 4D).

**Role of PTEN in gefitinib-mediated inhibition of Akt activation and production of angiogenic factors.** Akt activation is regulated by both phosphorylation at serine-473 and PI3

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**Figure 1.** Effect of EGF on the cell growth of three HCC cell lines, HCC3, CBO12C3, and AD3: 5.0 × 10⁴/well were incubated with or without 10 ng/mL EGF in 48-well plates. The cells were counted at the times indicated. Points, mean of triplicate wells; bars, SD.

**Figure 2.** Effect of gefitinib on angiogenesis induced by HCC cell lines in the mouse dorsal air sac assay. A, representative photographs of assay chambers containing HCC3, CBO12C3, or AD3 cells, with or without gefitinib at 75 or 150 mg/kg/d. Arrowhead, newly formed vessels, with characteristic zigzagging lines. Gefitinib was given p.o., daily, for 5 days. B, quantitative analysis of tumor angiogenesis induced by three HCC cell lines and its inhibition by gefitinib. The angiogenic responses were determined by counting the number of new blood vessels ≥3 mm in length as shown in (A). Columns, mean of five or six mice; bars, SD. *, P < 0.05; **, P < 0.01 compared with controls using the two-tailed Mann-Whitney U test.
binding to pleckstrin homology domain of Akt. The level of cellular
PIP3 is controlled by PI3K and the lipid phosphatase, PTEN (44).
We compared the expression of PI3K and PTEN in the three cell
lines in the presence or absence of EGF. In the absence of EGF,
all three cell lines expressed similar levels of the PI3K catalytic
subunit, p110, but HCC3 and CBO12C3 expressed rather higher
levels of the regulatory subunit, p85, than AD3 (Fig. 5A). Quan-
titative analysis showed that PTEN was expressed in AD3 at
about half the level in HCC3 or CBO12C3, both in the absence and
presence of EGF (Fig. 5A). This difference in PTEN expression
between AD3 and the other two cell lines was consistently found in
repeated experiments with independently cultured cell lines (data
not shown).

Because gefitinib had been found to be a much less effective
inhibitor of Akt activation and angiogenic factor production in
AD3 cells than in HCC3 cells (Fig. 3B), we next examined whether
the lower expression of PTEN in AD3 could be related to this.
In HCC3 cells transfected with PTEN siRNA, the
PTEN gene was very effectively silenced (see Fig. 5B); Akt was found to be
phosphorylated in the absence of EGF, but not in mock-
transfected cells; and Akt phosphorylation was much less sensitive
To inhibition by gefitinib (Fig. 5C). We then looked at cellular
levels of CXCL1 and VEGF. The cellular levels of CXCL1, but not
VEGF, in PTEN siRNA-transfected HCC3 cells were about half
those in mock-transfected cells (Fig. 5D) and the production of
both VEGF and CXCL1 in response to EGF became insensitive to
inhibition by gefitinib.

In Figure 3. Effect of gefitinib on the EGF-induced production of angiogenic factors and phosphorylation of EGFR, Akt, and ERK1/2 in HCC cell lines. A, 2.5 x 10^5 cells per well were incubated in the presence or absence of 10 ng/mL EGF, with or without different doses of gefitinib, in 24-well plates for 24 hours. VEGF and CXCL1 were measured in culture supernatants with an ELISA kit. Columns, mean for triplicate wells; bars, SD. *, P < 0.05; **, P < 0.01 compared with treatment with EGF alone using the two-tailed Student’s t test. B, the cells were stimulated with 10 ng/mL EGF for 15 minutes with or without gefitinib at the concentrations indicated. Protein extracts were resolved by Western blot analysis and probed with various antibodies. Activities of phosphorylated EGFR, Akt, and ERK1/2 were normalized to their nonphosphorylated form when each activity of phospho-EGFR, phospho-Akt, and phospho-ERK1/2 in the presence of EGF without gefitinib in three cell lines was normalized as 1.0.

Increased EGFR gene copy number in HCC cells. Cappuzzo
et al. (22) reported that gene amplification and copy number of
EGFR gene in association with Akt activation is closely associated
with drug sensitivity on therapeutic efficacies of gefitinib in
NSCLC. We examined if EGFR gene was amplified in liver cancer
cell lines, HCC3 and AD3 by FISH analysis. HCC3 and AD3 cells
showed increased copy number of EGFR gene compared with
normal spleen cells (Fig. 6). However, EGFR gene amplification
was not observed in both HCC3 and AD3. HCC3 cells were near
triploid with 2.7 ± 0.9 copies of chromosome 11 per cells and
61.3 ± 18.4 of chromosome per cells. AD3 cells were near
tetraploid with 3.7 ± 0.9 copies of chromosome 11 per cells and
77.8 ± 18.3 of chromosome per cells. We found that both cell
lines increased copy number of EGFR gene; however, there was
no apparent difference in both gene amplification and copy
number of EGFR in HCC3 and AD3.

Discussion
We have previously reported that gefitinib inhibits EGF-induced
angiogenesis both in vitro and in vivo (36, 37). In this study, we have
shown for the first time that tumor-induced angiogenesis in the
mouse dorsal air sac assay can be blocked by gefitinib treatment.
However, when different HCC cell lines were used in this model
to induce angiogenesis, its susceptibility to inhibition by this drug
was found to vary. Angiogenesis induced by HCC3 cells was most
susceptible to inhibition by gefitinib. Angiogenesis induced by
CB012C3 cells was also inhibited by gefitinib, but at higher doses of the drug. In contrast, angiogenesis induced by AD3 was relatively resistant to inhibition by gefitinib. We first examined expression of which angiogenic factor was augmented by exogenous addition of EGF in HCC cell lines by membrane-bound antibody array technology. Of various angiogenesis-related factors, we observed up-regulation of VEGF and CXCL1 genes by EGF (data not shown), and we focused on these two potent angiogenic factors. In all HCC lines, EGF was found to enhance cellular production of both VEGF and CXCL1. The production of these potent angiogenic factors was also more effectively inhibited by gefitinib in HCC3 and CB012C3 cells than that in AD3 cells. This suggests that the inhibitory effect of gefitinib on HCC-induced angiogenesis might be due to its effect on the production of such angiogenic factors. The production of angiogenic factors, including VEGF and IL-8, is often enhanced by EGF, TGF-α, and other cytokines in tumor cell lines (25, 38, 45–48). Consistent with these previous studies, the production of both VEGF and CXCL1 increased 1.6- to 3.2-fold with EGF treatment in all three HCC cell lines. The fact that gefitinib was a much less effective inhibitor of VEGF and CXCL1 production in AD3 than in HCC3 and CB012C3 cells suggests that the EGF induction mechanism in AD3 cells may be refractory to gefitinib. Our previous studies suggested two pathways by which gefitinib could exert its antiangiogenic effects: (a) EGF/TGF-α up-regulates the expression of angiogenic factors by cancer cells themselves, resulting in the induction of angiogenesis by a paracrine mechanism, and this process is inhibited by gefitinib; or (b) EGF/TGF-α induces angiogenesis through direct interaction with EGFRs on endothelial cells and this process is inhibited by gefitinib (36, 37). In our studies of EGF-induced neovascularization in the mouse cornea, we obtained evidence supporting the latter pathway as gefitinib inhibited activation of EGFR in the new vessels themselves (37). The results of our present study suggest that the former pathway is a more likely candidate because gefitinib inhibited the production of both VEGF and CXCL1 by HCC cells.

While studying the mechanism underlying the different inhibitory effects of gefitinib on VEGF and CXCL1 production by HCC cell lines, we found that the drug had differential effects on Akt activation. Whereas EGF treatment of HCC3 and CB012C3 cells resulted in the phosphorylation of EGFR, Akt, and ERK1/2, and this was highly susceptible to inhibition of gefitinib. In AD3 cells, gefitinib also inhibited the phosphorylation of EGFR and ERK1/2 in response to EGF, but Akt seemed to be constitutively active in the absence of EGF, and phospho-Akt levels in AD3 cells were less susceptible to the effects of gefitinib. Interestingly, LY294002, a selective inhibitor of PI3K, blocked production of VEGF and CXCL1 in HCC3, but not that in AD3, whereas U0126, an ERK1/2 inhibitor, did not have this selective inhibitory effect. Taken together, these different effects of gefitinib on the induction of angiogenesis and the production of angiogenic factors suggest an important role for the Akt pathway. However, as ERK1/2 and PI3K inhibitors also reduced the production of angiogenic factors, these signaling pathways may also play a role in the antiangiogenic effects of gefitinib.

Recent studies have shown that a mutation in exons 18 to 21 of the EGFRe gene is closely associated with a clinical response to gefitinib in NSCLC patients (18, 19) and that the proliferation of NSCLC cells carrying such EGFRe mutations is highly sensitive to gefitinib in vitro (18). We, however, could not observe any mutation in exons 18 to 21 of the EGFRe gene in three lines used in this study (data not shown). Cappuzzo et al. (22) have reported that EGFRe gene amplification and increased copy number are important to limit therapeutic efficacy of gefitinib in lung cancer. However, there was no difference in gene amplification and copy number of EGFRe in both HCC3 and AD3 cells. Therefore, altered expression of angiogenic factors and Akt activations between two lines might not be directly associated with gene amplification of EGFRe.

In this study, AD3 cells showed both constitutive Akt activation and reduced levels of PTEN compared with HCC3 and CB012C3 cells (Figs. 3B and 5A). It has been shown that phospho-Akt was recognized as a risk factor for early disease recurrence and poor prognosis of HCC (49). We have previously reported that sensitivity to gefitinib in NSCLC cell lines is associated with EGFR signaling pathways involving Akt and ERK1/2 and is closely linked with cell growth and apoptosis; activation of Akt and/or ERK1/2 was most susceptible to inhibition by gefitinib in one of the nine NSCLC cell lines, which carries a mutation in the EGFR catalytic domain (20). Both PI3K and PTEN are closely associated with activation of Akt, and reducing PTEN levels might be expected to change both Akt activation in response to EGF and its
sensitivity to gefitinib. Reducing PTEN gene expression in HCC3 cells using PTEN siRNA indeed decreased the sensitivity of both Akt activation and the production of VEGF and CXCL1 to gefitinib (Fig. 5). Another study has shown that loss of PTEN expression contributed to resistance to gefitinib independently of receptor kinase signaling, in EGFR-expressing tumor cells (50). These findings support our current evidence for a role of constitutive Akt activation in the gefitinib resistance shown by angiogenesis induced by AD3 cells. However, further studies will be required to understand exactly how constitutive Akt activation is associated with the sensitivity or resistance of EGFR signaling to gefitinib.

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Figure 5. Comparison of PI3K and PTEN protein levels in three HCC cell lines and effects of depleting PTEN using siRNA in HCC3. A, HCC cells were cultured with or without 10 ng/mL EGF for 24 hours. Protein extracts were resolved by Western blot analysis with anti-PI3K p110, anti-PTEN, or anti-β-actin antibodies. Levels of PTEN expression were measured by densitometry and normalized to the β-actin levels in each cell line. Columns, mean of triplicate experiments; bars, SD. B, HCC3 cells were transfected with PTEN siRNA, and after 24 hours PTEN levels were determined by Western blot analysis. Levels of PTEN expression were normalized to β-actin levels in each cell line. C, effect of PTEN siRNA on Akt phosphorylation in HCC3. Twenty-four hours after transfection with siRNA (100 nmol/L), HCC3 cells were stimulated with 10 ng/mL EGF for 15 minutes, with or without gefitinib at the concentrations indicated. Phosphorylated Akt levels were normalized to nonphosphorylated Akt levels in the presence of EGF but without gefitinib. D, effect of PTEN siRNA on inhibition of the EGF-induced production of VEGF and CXCL1 by gefitinib in HCC3. Cells (2.5 × 10^4/well) were incubated in the presence or absence of 10 ng/mL EGF with or without different doses of gefitinib in 24-well plates for 24 hours. Columns, mean of triplicate wells for nontransfected (closed), mock-transfected (hatched), and PTEN siRNA-transfected cells (open); bars, SD. *, P < 0.05; **, P < 0.01 compared with treatment with EGF alone using the two-tailed Student's t test.
References


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if $M = +0.27$ and $L = -0.16$ and the normal differential is 65 per cent M and 35 per cent L, then

$$0.65 (+0.27) + 0.35 (-0.16) = +0.12,$$

a figure identical to the observed +0.12 for normal leukocytes.
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