Conclusion

1. Lactoferrin has a growth-stimulatory activity for lymphoma cell lines in serum-free medium, and this effect is somewhat different from that of transferrin.
2. The growth-stimulatory activity of lactoferrin and transferrin for lymphoma cell lines is enhanced by the addition of iron to iron-free medium.
3. Lactoferrin has a marked growth-inhibitory activity for all adhesive cell lines tested, while transferrin has a growth-stimulatory or small growth-inhibitory activity.
4. $^{59}Fe^{3+}$-saturated lactoferrin binds gradually to lymphoma cell lines at 0°, while the binding of $^{59}Fe^{3+}$-saturated transferrin is more rapid.
5. $^{59}Fe^{3+}$-saturated lactoferrin associated with lymphoma cell lines at 37° reaches a plateau within 40 min, while the associated counts from $^{59}Fe^{3+}$-saturated transferrin continued to rise almost linearly over a period of 120 min.
6. $^{59}Fe^{3+}$-saturated lactoferrin displays K_d values of about 10^{-6} M with more than 10^6 binding sites per lymphoma cell. On the other hand, K_d values for $^{59}Fe^{3+}$-saturated transferrin are about one order lower at 10^{-7}–10^{-8} M and the receptor numbers are less than 10^6 sites per cell.
7. Lactoferrin binds to lymphoma cell lines without competition from transferrin.

[28] Biological Activity of Human Plasma Copper-Binding Growth Factor Glycyl-L-histidyl-L-lysine

By LOREN PICKART and STEVE LOVEJOY

Background and Preparation of GHL and GHL-Cu

Biological Actions of GHL and GHL-Cu

Glycyl-L-histidyl-L-lysine : copper(II) (GHL-Cu) is a growth factor isolated from human plasma. The peptide portion of the complex, GHL (glycyl-L-histidyl-L-lysine), has an affinity for copper(II) equivalent to that of the copper transport site on albumin and addition GHL to culture medium presumably results in a significant conversion into GHL-Cu by

chelation of ionic copper from the culture medium. GHL is used for *in vitro* culture of a diverse variety of cells and organs[1-3] where it appears to function as a transporter of ionic copper(II) and possibly other transition metal ions in serum-free or low-serum media.[4,5] In addition, GHL-Cu possesses significant superoxide dismutase-like activity (rate constant about 5×10^8 or about 20 to 25% of activity of enzymatic Cu,Zn-superoxide dismutase on a molar basis), an activity associated with tissue-protective and antitrauma effects and which may serve as the basis of some of GHL's diverse biological actions. In general, GHL works best with cell cultured on biological substrata (e.g., collagen, fibronectin) and often in cases where GHL gives a marginal effect, the use of GHL-Cu produces a marked response. Rapidly growing cells produce GHL or a similar peptide and recent work suggests a physiological role for GHL-Cu related to the processes of wound healing and tissue repair (e.g., angiogenesis, neurogenesis, chemoattraction of capillary endothelial and mast cells, acceleration of wound healing).

Structure–Activity Relationships in GHL and GHL-Cu

Physicochemical studies using a variety of techniques have indicated that GHL binds copper(II) to form a tridentate, triaza complex with copper being chelated to the α-amino nitrogen of glycine, the nitrogen in the amide bond between the glycyl and histidyl residues, and an unprotonated nitrogen in the imidazole ring of the histidyl residue (see Fig. 1).[5-11] The lysyl side chain is essential for activity. Analogs of that lack this residue such as glycyl-L-histidine and glycyl-L-histidyl-L-glycine form a similar

[1] L. Pickart, *In Vitro* **17,** 459 (1981).
[2] L. Pickart, *in* "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins" (B. Weinstein, ed.), p. 99. Dekker, New York, 1981.
[3] L. Pickart, *Lymphokines* **8,** 425 (1983).
[4] L. Pickart, J. Freedman, J. Loker, W. J. Peisach, C. M. Perkins, R. E. Stenkamp, and B. Weinstein, *Nature (London)* **288,** 715 (1980).
[5] J. Fernandez-Pol, *in* "Microbiology 1983" (D. Schlessinger, ed.), p. 313. American Society of Microbiology, Washington, D.C., 1983.
[6] S. Lau and B. Sarkar, *Biochem. J.* **199,** 649 (1981).
[7] J. Freedman, L. Pickart, B. Weinstein, W. B. Mims, and J. Peisach, *Biochemistry* **21,** 4540 (1982).
[8] E. Y. Kwa, B. S. Lin, N. J. Rose, B. Weinstein, and L. Pickart, *Pept. Struct. Funct.* **8,** 805 (1983).
[9] J. P. Laussac, R. Haran, and B. Sarkar, *Biochem. J.* **209,** 533 (1983).
[10] C. Perkins, R. Stenkamp, N. Rose, B. Weinstein, and L. Pickart, *Inorg. Chim. Acta* **67,** 93 (1984).
[11] M. J. A. Rainier and B. M. Rode, *Inorg. Chim. Acta* **92,** 1 (1984).

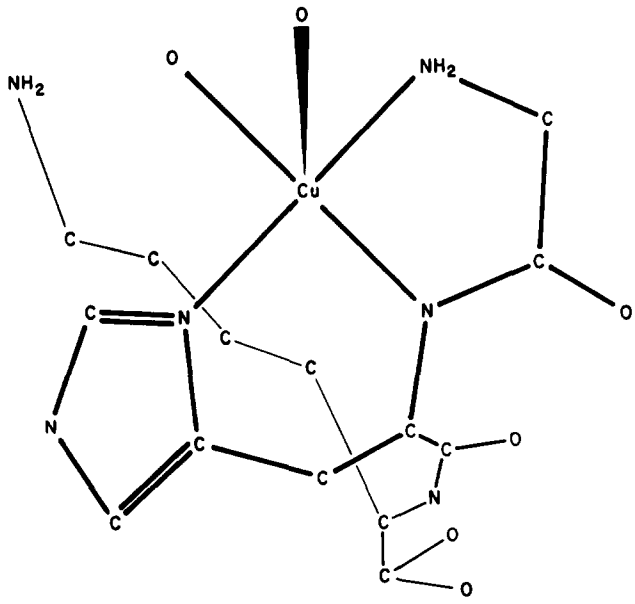

FIG. 1. Proposed structure of GHL-Cu in solution. This figure is based on X-ray analysis of GHL-Cu crystals and finds the copper ion bound to three nitrogen of GHL plus two oxygens in water. Other procedures (proton magnetic resonance, electron spin resonance, electron spin echo, minimum energy computer modeling) suggest essentially the same structure persists in solution. However, it is probable in physiological milieu that an amino acid such as histidine or cysteine displaces an oxygen and binds to the fourth equatorial bonding orbital of copper.

complex with copper(II)[8,11] but lack bioactivity.[12-14] Biological activity is retained after attachment of hydrophobic groups (e.g., octyl, benzyl) to the carboxyl-terminus of GHL.[15]

Affinity measurements indicate that GHL-Cu should be a reasonably stable complex *in vivo* under physiologic conditions. GHL has an amino acid structure similar to the copper ion transport site on human albumin[4] and has an affinity for Cu(II) equivalent to that of the copper ion transport site on plasma albumin (pK 16.2).[6] Measurements of the pK between GHL and copper(II) have given pK values of 14.9,[16] 16.2,[11] and 16.4[6] by potenti-

[12] L. Pickart and M. M. Thaler, *FEBS Lett.* **104,** 119 (1979).
[13] M. V. Williams and Y. Cheng, *Cytobios* **27,** 19 (1980).
[14] T. Poole and B. Zetter, *Cancer Res.* **43,** 5857 (1983).
[15] S. Lovejoy, Ph.D. thesis. Department of Chemistry, University of Washington, Seattle, Washington, 1985.
[16] P. M. May, J. Whittaker, and D. R. Williams, *Inorg. Chim. Acta* **80,** L5 (1983).

ometric titration, 16.3 by electron spin resonance spectroscopy,[11] and 16.5 with a copper-ion selective electrode.[17] It is likely GHL-Cu is even more stable than these pK values suggest since the fourth (unbound) equatorial orbital on copper in GHL-Cu can bind to other ligands and further stabilize the complex. For example, the pK of the GHL-Cu–histidine complex is 29.0.[6]

Preparation and Handling of GHL and GHL-Cu

GHL is widely available from commercial sources. Detailed information on the synthesis of GHL and analogs plus copper(II) complexes of these compounds is available in published theses by Lovejoy,[15] Loker,[18] Perkins,[19] and Kwa.[20] Commercial GHL is about 95% pure and useful for most cell culture purposes, but often includes small amounts of mildly neurotoxic materials (as measured by behavior after intracranial injection, tail flick assays, and gripping ability of mice on spinning disks). Most of this material can be removed by dissolving GHL in glass-distilled water (50 mg/ml), centrifuging at 20,000 g for 1 hr at 3°, then lyophilizing the supernatant. This removes poorly water-soluble material (probably GHL that was not completely deblocked of protecting groups during the final synthetic step). The supernatant is lyophilized, then passed through a Sephadex G-10 column at 3° in a solvent of 0.5% acetic acid in glass distilled water. The main peak that elutes behind the solvent front (monitored by absorption at 254 nm) is lyophilized to dryness.

GHL-Cu is prepared by combination of purified GHL with equimolar cupric acetate, followed by neutralization with 0.1 N sodium hydroxide, and centrifugation at 5000 g for 30 min at 3° to remove insoluble material [usually excess copper(II) as its hydroxide]. The supernatant is passed through a G-10 column in a solvent of glass-distilled water and the elution peak absorbing at 600 nm collected and lyophilized to obtain GHL-Cu. Crystalline GHL-Cu is prepared by dissolving purified GHL (30 mg, 88 μmol) in an aqueous solution copper(II) acetate (0.3 ml, 0.3 M). Ethanol (1.26 ml) is added and vessel walls scratched to initiate crystallization of dark blue-purple crystals. The mother liquor is decanted and the crystals

[17] A. Avdeef, L. Pickart, and B. Weinstein, unpublished observations.

[18] W. J. Loker, Ph.D. thesis. Department of Chemistry, University of Washington, Seattle, Washington, 1981.

[19] C. Perkins, Ph.D. thesis. Department of Biological Structure, University of Washington, Seattle, Washington, 1982.

[20] E. Kwa, Ph.D. thesis. Department of Chemistry, University of Washington, Seattle, Washington, 1983.

dissolved by addition of distilled water (0.2 ml). Ethanol (0.4 ml) is slowly introduced to reach a cloud point. After standing, dark purple-blue octahedral crystals formed that were isolated by decanting the mother liquor.[10] Microanalysis to determine amino acid and copper content confirms the composition of the product.

Both GHL and GHL-Cu are stable for more than 4 years when stored at −20° in a desiccator over $CaCl_2$. GHL is hygroscopic, hence must be weighed rapidly. Aqueous solutions (50 mg/ml) in buffer (pH 7.4) are stable at 3° for at least 6 months. The use of reasonably concentrated solutions (>1 mg/ml) during sterile filtration and for dilution is recommended because of absorption to glass and plastic surfaces at low (<20 μg/ml) concentrations. Often GHL is more effective on sparse cell cultures; this may reflect endogenous synthesis of adequate quantities of GHL by rapidly growing cells.[14]

The Uses of GHL in Culture Systems

Neuronal Cells

GHL facilitates the growth of cultured central and peripheral neurons plus promoting axonal and dendrite outgrowth. The use of GHL in low-serum medium also promotes the growth and survival of neurons while decreasing the glial cell content, thus markedly increasing the neuron to glial cell ratio in culture.

For cultivation of chick cerebral neurons,[21,22] a medium composed of Eagle's minimum essential medium (MEM) supplemented with 500 mg% glucose, 1% fetal calf serum, and GHL (200 ng/ml) is used. Embryonic cerebral cells are obtained from 7-day-old cerebral hemispheres and plated onto Petri dishes covered with denatured collagen and covered with medium. After 4 days in this medium, there is strong nerve fiber differentiation and axonal outgrowth while glial cell growth is greatly suppressed resulting in an essentially pure neuronal culture.

GHL has also been used to stimulate neuronal outgrowth of chick embryo ganglion trigeminale cells cultured in a similar medium but with 5% placental serum and GHL (10 ng/ml).[23]

[21] M. Sensenbrenner, G. G. Jaros, G. Moonen, and P. Mandel, *Neurobiology* **5**, 207 (1975).
[22] M. Sensenbrenner, G. G. Jaros, G. Moonen, and B. J. Meyer, *Experientia* **36**, 660 (1980).
[23] G. Lindner, G. Gross, W. Halle, and P. Mandel, *Z. Mikrosk.-Anat. Forsch.* **93**, 820 (1979).

Glomerular Kidney Cells

Primary glomerular kidney cells may be cultured in media containing high concentrations of calf serum or alternately on fibronectin in a defined medium containing GHL.[24] Use of GHL as the kidney cell growth factor eliminates some of the dedifferentiation of glomerular cells observed with serum-containing medium.

Young guinea pigs (Hartley, 300 g) are used as the source of glomeruli, of which 2000 are added to 9.6 cm^2 wells in 2 ml medium. Culture medium consists of Waymouth's MB 752/1 medium supplemented with sodium pyruvate (1%), nonessential amino acids (1%, Gibco), penicillin–streptomycin (100 units/ml), GHL (50 μg/ml), and fibronectin (10 μg/ml). During the several days it takes glomeruli to attach, 1 ml of medium in the well is replaced daily with fresh medium. After attachment, cells are fed three times weekly. Four distinct types of well-differentiated glomerular cells [I, II(a), II(b), II(c)] are observed under these culture conditions with type II(a) becoming predominant.

Organ Culture on Collagen Pads

GHL is used for the culture of a variety of organs. Organ pieces (human liver,[25,26] human kidney,[27] human placental tissue,[28] normal and neoplastic endometrium,[29] plus human kidney cells[27]) are cultured on gelatin (collagen) foam slices (Spongostan, Ferrosan, Sweden) in plastic Petri dishes using Parker's 199 medium supplemented with GHL (20 ng/ml). Tissue explants of 1 to 1.5 mg are used. In this medium, liver explants demonstrate linear production of export proteins (albumin, orosomucoid, α_1-antitrypsin, protein HC) for 10 days while kidney explants remain viable for 20 days and kidney cells for 35 days.

In Vitro Model of Fibroplasia

This *in vitro* model of fibroplasia permits simultaneous quantification of fibroblast proliferation, migration, and collagen synthesis in a GHL-

[24] T. D. Oberley, P. J. Murphy, B. W. Steinert, and R. M. Albrecht, *Virchows Arch. B* **41,** 145 (1982).

[25] S. Eriksson, R. Alm, and B. Astedt, *Biochim. Biophys. Acta* **542,** 496 (1978).

[26] L. Tejler, S. Eriksson, A. Grubb, and B. Astedt, *Biochim. Biophys. Acta* **542,** 506 (1978).

[27] B. Astedt, G. Barlow, and L. Holmberg, *Thromb. Res.* **11,** 149 (1977).

[28] L. Holmberg, I. Lecander, B. Persson, and B. Astedt, *Biochim. Biophys. Acta* **544,** 128 (1978).

[29] L. Svanberg and B. Astedt, *Experientia* **35,** 818 (1979).

based culture medium and facilitates the study of the influence of various healing and inflammatory factors on the events in fibroplasia.[30]

Fibroblasts are prepared from tendon plugs (2 mm diameter) prepared from flexor digitorium profundus tendons removed from the long digit of female Leghorn chickens. Plugs are placed in culture wells and first covered with 10 μl thrombin solution (2 mg/ml in DMEM), then 50 μl fibrinogen solution (3 mg/ml), allowed to clot for 30 min at 37° in a humidified atmosphere, then covered with 1 ml of medium used to maintain fibroblasts in a quiescent state. This medium consists of Dulbecco's modified Eagle's medium to which are added GHL (200 ng/ml), aprotinin (100 kIU/ml, Trasylol), and ascorbate (0.1 m*M*). After 48 hr, exogenous factors to be assayed (e.g., calf serum, platelet lysate) are added. Fresh ascorbate is added every 24 hr.

Fibroblast migration is quantified by measurement of cellular outgrowth from the tendon plug by planimetry of photomicrographs. Fibroblast proliferation was measured as pulsed incorporation of [^{125}I]iododeoxyuridine (2000 Ci/mmol) into DNA in the presence of 10^{-5} *M* 5-fluorodeoxyuridine. Collagen synthesis is measured by the incorporation of [^{3}H]proline into cellular protein for 6 hr followed by treatment with cold 5% trichloroacetic acid to precipitate protein and radioactivity of the precipitate determined. Treatment with collagenase to lyse collagen followed by another trichloroacetic acid precipitation permits a correction for proline incorporation into noncollagen protein.

Immunologically Related Cells

The inclusion of GHL in culture media used for assay of cellular immune function often permits the elimination or a marked reduction in the serum requirement. For example, good lymphocyte transformation responses can be obtained in medium with 20 ng/ml GHL, eliminating the normally required 5 to 10% serum in medium. This serum reduction can minimize many of the experimental complications associated with serum use. For eosinophils, macrophages, and mast cells, the serum requirement is reduced from 10 to 20% down to 1%.

Lymphocyte Transformation.[31] Lymphocytes obtained from spleen tissue of antigen-sensitized rats are preincubated with RMPI 1640 medium supplemented with 5% fetal calf serum for 45 min, washed 3 times with medium to remove serum, then cultured in medium supplemented with GHL (20 ng/ml), L-glutamine (290 μg/ml), and antibiotics. Lymphocyte

[30] M. F. Graham, R. F. Diegelman, and I. K. Cohen, *Proc. Soc. Exp. Biol. Med.* **176,** 302 (1984).

[31] A. Haque and A. Capron, *Nature (London)* **299,** 361 (1982).

transformation is performed in microtiter plates using 0.1 ml medium containing 0.5×10^6 cells. Antigens are added to the wells and after 4 hr pulsed with 1 μCi [^{3}H]thymidine. Cells are collected on a Titertek cell collector (Skatron) and incorporated radioactivity determined in a scintillation counter.

Eosinophil Preparations.[32,33] Eosinophils are collected from normal rats by rinsing peritoneal cavities in culture medium [Eagle's MEM, 1% heat-inactivated fetal calf serum, GHL (20 ng/ml)] plus 25 IU/ml calcium heparinate. Eosinophil-rich fractions are prepared by centrifugation at 1800 *g* at 4° and washed twice in the medium, then added to plastic Petri dishes for 2 hr at 37° in a 5% CO_2 atmosphere. The nonadherent cells are collected and pooled. This population consists of 47% eosinophils and 9% mast cells and is maintained in the GHL-containing medium during further purification steps and during assay procedures.

Macrophage Cytotoxicity.[34,35] Macrophage cytotoxicity toward ^{51}Cr-labeled schistosomula was determined by collecting normal rat macrophages by peritoneal washings. The cells were cultured in Eagle's MEM with GHL (20 ng/ml) and 1% heat-inactivated fetal calf serum at 37° for 2 hr. Nonadherent cells are removed by three washings with medium. The adhering macrophage cells are cultured overnight in the medium. Macrophages are activated by incubation with MEM medium containing 20% serum from infected rats for 3 to 6 hr, after which the labeled schistosomula are added. Macrophage cytotoxicity is determined by the percentage of chromium released after an overnight incubation.[34]

A GHL-containing medium (Hanks–Wallace medium, 2 m*M* glutamine, 20 ng/ml GHL) is also effective for studies of IgE stimulation of human alveolar macrophages. The endpoint in IgE stimulation is the release of lysosomal β-glucuronidase and neutral proteases.[35]

Mast Cell Function.[36] Mast cell degranulation induced by nonspecific degranulators or anaphylactic antibodies reacting with antigen is quantified by incubation of rat or mouse mast cells in a medium consisting of Eagle's MEM, GHL (20 μg/ml), calcium heparinate (50 U/ml), and glutamine (2 m*M*). The mast cells are incubated for 30 min at 37° in the medium plus 2 μCi [^{3}H]serotonin for 10^6 cells, then washed 3 times to remove unincorporated medium. The percentage of radioactivity released

[32] M. Capron, J. Rousseaux, C. Mazingue, H. Bazin, and A. Capron, *J. Immunol.* **121,** 2518 (1978).

[33] M. Capron, A. Capron, G. Torpier, H. Bazin, D. Bout, and M. Joseph, *Eur. J. Immunol.* **8,** 127 (1978).

[34] M. Joseph, J. P. Dessaint, and A. Capron, *Cell. Immunol.* **34,** 247 (1977).

[35] M. Joseph, A. B. Tonnel, A. Capron, and C. Voisin, *Clin. Exp. Immunol.* **40,** 416 (1980).

[36] C. Mazingue, J. P. Dessaint, and A. Capron, *J. Immunol. Methods* **21,** 65 (1978).

into the medium by after addition of degranulating substances gives the measure of degranulation.

Hepatocytes and Hepatoma Cells

The viability of hepatocytes, when cultured in low-serum medium (0.5 to 1.0% fetal calf serum in 90% Eagle's MEM and 10% Swim's S-77 medium) is enhanced, and the growth of hepatoma cells (HTC_4 or AH-130) stimulated, by addition of GHL (20 ng/ml).[37-39] This action on hepatoma cells is more pronounced when the GHL is prechelated to copper(II) and iron(II) suggesting the primary role of GHL in this system is transition metal ion transport.[40] The addition of GHL (2 ng/ml) to isolated rat hepatocytes cultured in L-15 medium results in a 27% increase in α_1-macroglobulin synthesis while reducing transferrin synthesis 29%.[41]

Mast Cell Migration Assay

GHL is a potent chemoattractant for mast cells.[14] Plastic tissue culture dishes are pretreated with a 1% aqueous solution of bovine serum albumin, then 2.5 ml of 2.4% agarose is mixed with a 2× concentrated tissue culture medium 199 supplemented with 0.2% albumin at 50°, is poured into the dishes. After cooling to room temperature, a gel forms. With an Ouchterlony punch and template, 3-mm wells are punched 3 mm apart in a concentric circle around a center well. Rat mast cells (3×10^4) are added to the center well in 10 μl medium and the outer wells receive solutions of GHL or other chemoattractant substances. After 4 hr, the dishes are fixed in 10% formalin, dried, and stained with Bismark brown. Migration patterns are quantified using a stage micrometer at 20–30× magnification to measure linear distance.

Ascaris suum and Litomosoides carinii Larvae

The culture of these parasitic organisms through their third to fourth stage of morphogenesis can be achieved using serum supplemented medium. However, their cultivation in serum-free GHL-containing medium greatly facilitates the collection of stage-specific somatic and excretory/

[37] L. Pickart and M. M. Thaler, *Nature (London) New Biol.* **243,** 85 (1973).
[38] L. Pickart, L. Thayer, and M. M. Thaler, *Biochem. Biophys. Res. Commun.* **54,** 562 (1973).
[39] T. Aoyagi and H. Umezawa, *Proc. FEBS Meet.* **61,** 89 (1980).
[40] L. Pickart and M. M. Thaler, *J. Cell. Physiol.* **102,** 129 (1980).
[41] F. M. Fouad, M. A. E. Fattah, R. Scherer, and G. Ruhenstroth-Bauer, *Z. Naturforsch.* **36c,** 350 (1980).

secretory antigens that are used for the development of antiparasite vaccines.

Ascaris suum larvae are collected from the lungs of rabbits 10 days after infection with 100,000 embryonated eggs. Third-stage larvae are incubated in Medium 199 supplemented with GHL (14 ng/ml), penicillin (300 units/ml), and streptomycin (0.3 mg/ml) under an atmosphere of N_2–CO_2–O_2 (90 : 5 : 5) at 37°. After 12 days the larvae grow in length and moult to the fourth developmental stage. Larval proteins isolated from used growth medium are collected, and a larval antigen isolated that produces immunological protection from *Ascaris* infection.[42,43]

Inocula of *L. carinii* (stage 3) are recovered from rats 7 days after exposure of rats to mites infected with the nematode. Larvae are collected aseptically by lavaging the pleural cavities of the rats with warm Lebovitz's L-15 medium. The larvae progress from stage 3 to stage 4 after 4 to 10 days culture in L-15 medium containing 2 μg/ml GHL.[44]

T-Strain Mycoplasma (Ureaplasma urealyticum)

The incorporation of GHL into the basic indicator broth used for clinical detection of the T-strain mycoplasma *Ureaplasma urealyticum* associated with urinary tract infections results in an increased initial growth rate and higher final titer of the organism. This improved detection broth consists of PPLO broth (2.1 g, Difco), yeast extract (0.1 g, Difco), 0.4% bromothymol blue solution (1 ml), horse serum (10 ml), urea (0.025% w/v), and GHL (20 ng/ml).[45]

Coelomomyces punctatus

GHL added to basic media stimulates the initial growth and development of the fungi, *Coelomomyces punctatus,* known to parasitize and cause high levels of mortality in natural populations of mosquito larvae. The fungi are isolated from the gut of mosquito larvae of *Anopheles quadrimaculatus* and *Cyclops vernalis,* then cultured in Mitsuhashi–Maramorosch insect tissue culture medium which has been conditioned by growing Varma's *Anopheles stephensi* cells in it for 3 weeks, and supplemented with 20% fetal calf serum and GHL (20 ng/ml).[46]

[42] B. E. Stromberg, P. B. Khoury, and E. J. L. Soulsby, *Int. J. Parasitol.* **7,** 149 (1977).
[43] B. E. Stromberg, *Int. J. Parasitol.* **9,** 307 (1979).
[44] P. D. Nelson, D. J. Weiner, B. E. Stromberg, and D. Abraham, *J. Parasitol.* **68,** 971 (1982).
[45] J. A. Robertson, *J. Clin. Microbiol.* **7,** 127 (1978).
[46] J. M. Castillo and D. W. Roberts, *J. Invertebr. Pathol.* **35,** 144 (1980).

GHL as a Component of Growth-Promoting Mixtures

Primary Human Tumor Lines

The establishment of cells from primary human tumors (mammary, ovarian, cervical) for over than 3 months with a greater than 80% success rate was achieved using a medium composed of Earle's salts, MEM vitamins (1×), MEM nonessential amino acids (2×), MEM essential amino acids (4×), L-glutamine (4 m*M*), sodium pyruvate (1 m*M*), and 10–20% fetal calf serum plus the growth factors GHL (10 ng/ml), insulin (80 mIU/ml), transferrin (10 ng/ml), fetuin (5 μg/ml) and L-thyroxine (10^{-7} *M*).[47]

Rat Thyroid Follicular Cells

Rat thyroid cells are cultured in Ham's F12M medium and 0.5% calf serum plus six hormones; GHL (10 ng/ml), insulin (10 μg/ml), hydrocortisone (10 n*M*), transferrin (5 μg/ml), somatostatin (10 ng/ml), and thyrotropin (10 mU/ml). This combination produced diploid growth of the thyroid cell line for greater than 175 generations with maintenance of normal morphology, secretion of thyroglobulin, and ability to concentrate iodine from the culture medium.[48]

Human KB and HeLa Cells

The growth rate of the human tumor cell lines, KB and HeLa, cultured on growth-limiting levels (0.5%) of dialyzed fetal calf serum in Eagle's minimal essential medium plus nonessential amino acids (1×) was stimulated by the addition of GHL (250–500 ng/ml) and bovine serum albumin (fatty acid free, 6 mg/ml). These two additions produced a cellular growth rate in 0.5% serum equivalent to that normally obtained with 5% serum.[13]

Erythropoietin Production in Rats

In normal and hepatectomized rats, the intravenous injection of GHL (100 μg) produces about a 40% rise in erythropoietin, the hormone that increases red blood cell production.[49] This increase is similar to that pro-

[47] W. E. Simon and F. Holzel, *J. Cancer Res. Clin. Oncol.* **94,** 307 (1979).

[48] F. S. Ambesi-Impiombato, L. A. M. Parks, and H. G. Coon, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 3455 (1980).

[49] B. A. Naughton, G. K. Naughton, P. Lui, G. B. Zuckerman, and A. S. Gordon, *J. Surg. Oncol.* **21,** 97 (1982).

duced by about 500 μg glucagon. In this system, insulin administration was without effect.

These effects may reflect a linkage between GHL's growth and viability enhancing actions on liver and kidney cells and the production of erythropoietin by the fetal liver and adult kidney.

Uses of GHL-Cu

Induction of Angiogenesis Effectors and New Capillary Formation

For the induction of new capillary formation in the rabbit eye model, GHL-Cu is incorporated into a slow-release plastic polymer (Elvax), then inserted into a 2 × 3 mm surgical pocket in the lower cornea of New Zealand rabbits (2 to 3 kg).[50] The amount of GHL-Cu in the polymer is calculated to introduce 20 μg into the piece inserted into the corneal pocket. After 80 hr, budding of capillaries begins and by 7 to 8 days dense capillaries surround the implant.

GHL-Cu induces synthesis of proteins that act as potent chemoattractants toward capillary endothelial cells and a family of 5 to 6 angiogenic-related polypeptides with molecular weights ranging from 35 to 66 × 10^3 by 76 to 80 hr after application and immediately prior to endothelial cell migration influx.[51] These proteins are obtained from corneal tissue at this time by anesthetizing the rabbit, freezing the corneas with a dichloromethane spray, excising the tissues immediately underlying the GHL-Cu impregnated pellet, and storing them at −80° until use. An extract of this tissue is prepared by chopping the thawing samples into fragments about 1 mm^3 and incubating them overnight at 37° in Dulbecco's MEM or in phosphate-buffered saline. The volume of liquid is adjusted to obtain, after 15 hr of extraction followed by centrifugation (2000 *g* for 15 min), a supernatant containing 1.5 mg/ml protein.

The chemoattractant activity in this supernatant is assayed by determining the passage of cells across pores against a gradient of migration effector. The Boyden chamber has an upper well of 200 μl in size and a lower well of 40 μl which are separated by a poly(vinylpyrrolidone) nucleopore filter (Nucleopore Corp.) 13 mm in diameter precoated with type I collagen. A 5-μm micropore size filter is used for capillary endothelium. Both chambers are filled with DMEM with 1% fetal calf serum. About 10^5 cells in 100 μl medium are placed in the upper chamber and the migration

[50] K. Raju, G. Alessandri, and P. Gullino, *J. Natl. Cancer Inst. (U.S.)* **69,** 1183 (1983).
[51] K. Raju, G. Alessandri, and P. Gullino, *Cancer Res.* **44,** 1579 (1984).

effector (i.e., tissue proteins) placed in the lower chamber and incubated for 3 hr at 37° in 5% carbon dioxide. At the end of the migration, the cells are fixed in 10% formalin in PBS and stained with Wright's stain. Cells on the underside of the filter are counted in ×200 microscope fields.

The angiogenic related proteins in this supernatant are purified by passage through a gelatin : Sepharose 4B (Pharmacia) column (0.7 × 20 cm, 2 to 3 ml bed volume) in 0.02 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.4. After application the column is washed with the buffer to remove nonadhering proteins, then eluted with a solution containing 0.05 *M* sodium acetate and 1.0 *M* sodium bromide, pH 5.0. The eluate is dialyzed against 50 m*M* ammonium acetate to remove salts, then lyophilized. Proteins are reduced with 10% 2-mercaptoethanol then separated by SDS–polyacrylamide gel electrophoresis using a 3.3% spacer gel and a 5 or 7.5% separating gel.

Acceleration of Wound Healing

Administration of GHL-Cu accelerates the healing and closure of superficial wounds in rats, mice, and pigs.[52,53] For a wound in rats caused by the removal of a circular patch of skin (2 cm diameter), an effective procedure is the infiltration of 50 μg of GHL-Cu (in 50 μl phosphate buffered saline, pH 7.4) into the skin surrounding the wound margin after wounding, and 24 and 48 hr later. The enhancement of reepithelialization and wound closure becomes statistically significant by 15 days. At 25 days after surgery, 60% of the GHL-Cu treated wounds are fully healed while no controls are fully healed. In pigs, one treatment with GHL-Cu accelerated the healing of square (2.5 cm) wounds on the upper back in comparison with contralateral control wounds on the same animal. After 21 days, one treatment with GHL-Cu reduced the remaining wound size 64.9% (±22.1) in 14 wounds on eight pigs observed for this period ($p = 0.0023$ for pooled data). Pig wounds covered with autologous skin grafts or dehydrated pigskin responded best to GHL-Cu, but the healing of collagen pad covered wounds and uncovered wounds was also enhanced. Multiple treatments of pig wounds with GHL-Cu produced a more pronounced healing effect. In mice, daily swabbing of linear superficial incision wounds with GHL-Cu (100 μg/ml) produces a significant acceleration of wound closure by the fifth day.

This healing action presumably arises both from GHL-Cu's tissue-protective superoxide dismutase activity that minimizes tissue damage

[52] L. Pickart, *U.S. Patent Appl.* Serial Number 694,430, Jan. 24, 1985.

[53] L. Pickart, D. Downey, S. Lovejoy, and B. Weinstein, *Proc. Int. Conf. Superoxide Dismutase*, **4th,** Sept. 1985.

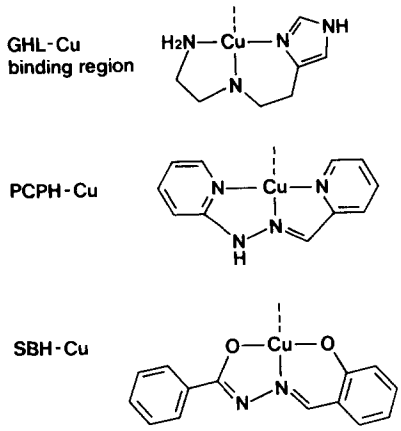

FIG. 2. Comparison of proposed structure of the GHL-Cu copper-binding region and the structures of PCPH-Cu and SBH-Cu. The PCPH-CU and SBH-Cu structures are based on X-ray data plus spectroscopic and nuclear magnetic resonance measurements. In all compounds a near-planar ring structure surrounds the copper. Other small ligands (amino acids, salts) are likely to bind to unsaturated bonding orbitals of copper.

after wounding and its chemoattractant actions on mast and capillary endothelial cells that produce an enhanced accumulation of these cells in the wound area; thusly stimulating neovascularization and nutrient flow into the wound.

Growth-Inhibitory Aroylhydrazone Analogs of GHL-Cu

Aroylhydrazone analogs, in which the copper-binding region is structurally similar to GHL-Cu, are potent mitotic inhibitors with antitumor and immunosuppessive actions (see Fig. 2).[54-58]

The most potent of these analogs, PCPH-Cu [pyridine-2-carboxyl-aldehyde-2'-pyridylhydrazonatocopper(II) dichloride] is prepared by reacting PCPH (0.40 g in 40 ml ethanol, Aldridge Chemical, Milwaukee, WI) with $CuCl_2 \cdot 2H_2O$ (0.34 g in 40 ml ethanol). On standing at room tempera-

[54] L. Pickart, W. H. Goodwin, W. Burgua, T. B. Murphy, and D. K. Johnson, *Biochem. Pharmacol.* **32,** 3868 (1983).
[55] L. Pickart, W. H. Goodwin, and W. Burgua, *J. Cell Biol.* **7A,** 175 (1983).
[56] D. K. Johnson, T. B. Murphy, N. J. Rose, W. H. Goodwin, and L. Pickart, *Biochem. Pharmacol.* **32,** 3868 (1983).
[57] A. A. Aruffo, T. B. Murphy, D. K. Johnson, N. J. Rose, and V. Shoemaker, *Inorg. Chim. Acta* **67,** L25 (1982).
[58] E. J. Blantz, F. A. French, J. R. Doamaral, and D. A. French, *J. Med. Chem.* **13,** 1124 (1970).

ture, green needlelike crystals are deposited, filtered off, washed with ethanol, and dried under vacuum.

For use, PCPH-Cu is dissolved in water (2 mg/ml) at 37°, then mixed with growth medium to obtain desired concentrations for testing. Glass pipets and glassware must be used because of the propensity of PCPH-Cu to absorb to plastics, a problem especially acute at concentrations below 1 ng/ml. For most cell lines, a concentration of 1 ng/ml produces a halving of DNA synthesis.

When used as an antitumor agent against fibrosarcoma MCA-1511 in mice, PCPH-Cu (100 μg in 0.05 ml aqueous buffer) is injected directly into the tumor twice weekly for 1 month. This results in the killing of about one-third of implanted tumors. Intraperitoneal administration is partially effective, but intravenous injection by tail vein is difficult due to the rapid development of necrosis at the infusion site. After 1 month of injection of PCPH-Cu into the tumor area, the mice in which the tumors have disappeared resume a normal pattern of weight gain and growth.

Salicylate benzoyl hydrazonate copper(II) (SBH-Cu) is a similar mitotic inhibitor, being about one-tenth as potent as PCPH-Cu. SBH-Cu is synthesized by dissolving benzoylhydrazone (20 mmol) in an ethanol : water mixture (1 : 3, v/v, 40 ml). A solution of salicylaldehyde (2.44 g, 20 mmol) in ethanol (20 ml) is added to the hydrazide solution with stirring, then placed on a steam bath for 20 min. When cooled to room temperature, crystals of salicylate benzoylhydrazone form are filtered off and dried under vacuum. Recrystallization from ethanol yields the pure product. Copper is complexed to the hydrazone by dissolving the hydrazone crystals (0.50 g, 2.1 mmol) in boiling 95% ethanol (20 ml). This solution was then added to a solution of copper(II) chloride dihydrate in boiling ethanol (20 ml) forming a deep green-brown solution. Cooling overnight yielded black, rodlike crystals that were filtered off, washed with ethanol (2 × 10 ml) and diethyl ether (2 × 10 ml), then dried under vacuum.

[29] Isolation of Human Erythropoietin with Monoclonal Antibodies

By RYUZO SASAKI, SHIN-ICHI YANAGAWA, and HIDEO CHIBA

Mature erythrocytes have a limited life span and cannot proliferate. The recruitment of these cells is achieved by differentiation and maturation of the erythroid precursor cells. All types of blood cells, including